Mechanisms of Cell Damage and Recovery in Cryopreserved Freshwater Protists

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Abstract.

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R. A. Fleck

Cryopreservation of biological tissues with storage in liquid or vapour phase nitrogen (LN) has frequently been adopted as the *in vitro* preservation method of choice. It offers a cost effective technique for the long-term maintenance of cultures and effectively guarantees genetic stability. To improve post-thaw viability levels and to preserve freeze-recalcitrant organisms, components of the cryogenic process which predispose and/or subject cells to stress and injury require to be determined. In this study markers of stress and cryoinjury were identified using flow cytometry and cryomicroscopy, they include: intracellular ice formation, cryoprotectant toxicity, osmotic shock (due to excessive cryodehydration), freeze-fracture events and morphological changes. Further studies investigated: inhibition of photosynthetic oxygen evolving capacity, products of lipid peroxidation, hydroxyl radical production, changes in antioxidant status [superoxide dismutase (SOD), catalase, peroxidase, glutathione reductase] and levels of protein and non-protein bound sulfhydryl groups (SH) in treated and untreated cells.

The morphological and physiological diversity of the algae influenced their responses to stresses and injury induced during cryopreservation. Compartmentalisation of ice nucleation contributed to the ability of *Enteromorpha intestinalis* (L.) Link CCAP 320/1 to survive freezing. In contrast, propagation of intracellular ice throughout the coenocytic alga *Vaucheria sessilis* (Vaucher) De Candolle ex Collins CCAP 745/1C enhanced its freeze-recalcitrance. Lipid deposition and inhibition of photosynthetic capacity on chilling were also detected in this alga.

An effective protocol, which avoided lethal cryoinjury, was developed for the previously freeze-recalcitrant alga *Euglena gracilis* Klebs CCAP 1224/5Z. This alga experienced non-lethal responses to the cryopreservation protocol including inhibition of photosynthetic oxygen evolution. Oxidative stress was also identified and the production of highly toxic hydroxyl radicals ($^{\cdot}OH$) was assessed using a novel non-destructive gas chromatographic technique. Cells were demonstrated to experience both lethal and non-lethal stresses. A mechanism contributing to the relative freeze-recalcitrance of *E. gracilis* is proposed involving increased SOD activity, which increases the availability of H_2O_2, thus contributing to the generation of the highly cytotoxic hydroxyl radical through Fenton chemistry. Elevated antioxidant activities were also detected for those enzymes which remove H_2O_2, these changes in antioxidant status, when poorly regulated, may in themselves contribute to oxidative stress and imbalances in their complex interactions may result in cryoinjury. Protection by the preferential oxidation of non-protein bound SH groups and a possible acclimation response enhancing protein SH levels were also detected in cryopreserved algae. In *Haematococcus pluvialis* Flotow CCAP 34/8 a regulated antioxidant response and high carotenoid composition were considered to contribute to freeze-tolerance.

Novel techniques (encapsulation/two-step cooling) and the use of exogenous antioxidants were investigated and these offer considerable scope for the preservation of presently freeze-recalcitrant protists. The benefits of using investigative rather than speculative approaches to develop cryoprotocols are discussed.

Mechanisms of Cell Damage and Recovery in Cryopreserved Freshwater Protists.

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Some moments that I've had

Some moments of pleasure..... (Kate Bush, 1993)

To my parents

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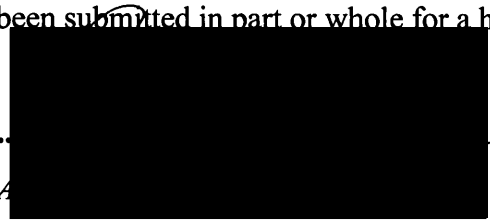
Declaration.

This thesis records the results of experiments carried out by myself in the Culture Collection of Algae and Protozoa, Institute of Freshwater Ecology, Windermere Laboratory and the School of Molecular and Life Science, the University of Abertay Dundee, under the supervision of Dr. E. E. Benson, Dr. J. G. Day and Dr. D. B. Bremner between November 1994 and October 1997. It is of my own composition and has not been submitted in part or whole for a higher degree.



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Roland A

Abbreviations.

A

Absorbance at X nm	A_x
American Type Culture Collection	ATCC
Analysis of variance	ANOVA

B

Blue Green medium	BG11
Bovine serum albumin	BSA

C

Chlorophyll *a*	Chl. *a*
Circa	*ca.*
Cubic ice	I_c
Culture Collection of Algae at the University of Texas at Austin	UTEX
Culture Collection of Algae and Protozoa	CCAP

D

Degrees Celsius	°C
Deionised water	dH_2O
Deoxyribonucleic acid	DNA
5,5′-dithiobis-(2-nitrobenzoic acid)	DTNB
Desfferrioxamine	DesF
Differential scanning calorimetry	DSC
Dimethylsulfoniopropionate	DMSP
Dimethylsulphoxide	DMSO
Diatom Medium	DM

E

Electron microscopy	EM
Electron paramagnetic resonance	EPR
Equilibrium freezing point	T_m
Ethylenediaminetetraacetic acid	EDTA
Ethylene glycol	EG
European Culture Collection Organisation	ECCO

F

Flurorescein diacetate	FDA
Forward light scatter	FSC

G

Gas chromatography	GC
Glass transition temperature	T_g
Glutathione peroxidase	GSH-Px
Glutathione reductase	GR
Gram	g

H

Hexagonal ice	I_h
Homogeneous ice nucleation temperature	T_h
Hour	h.
Hydroxyl free radical	$^{\cdot}OH$

I

Industrial methylated spirits	IMS
International Plant Genetic Resources Institute	IPGRI

J

Jaworski's Medium	JM

K, L

Lauryl sulphate (sodium dodecyl sulphate)	SDS
Liquid nitrogen	LN
Litre	l

M

Malondialdehyde	MDA
Melting temperature	T_m
Methanol	MeOH
Microbial Culture Collection National Institute for Environmental Studies (Japan)	NIES
Microgram	µg
Microlitre	µl
Micrometre	µm
Millilitre	ml
Millimetre	mm
Millimolar	mM
Millimoles per.litre	mmol/l
Minute	min.
Molar	M
Moles per.litre	mol/l

N

Nanometer	nm
Nanomole	nmol
Nicotinamide-adenine dinucleotide	NAD
Nicotinamide-adenine dinucleotide (reduced NAD)	NADH
Nicotinamide-adenine dinucleotide phosphate	NADP
Nicotinamide-adenine dinucleotide phosphate (reduced NADP)	NADPH
Nitrotetrazolium blue	NBT
Not determined	N/D
Number	No.

O

Oxidised glutathione	GSSG

P

Parts per thousand	‰
Parts per million	ppm
Polyethylene glycol	PEG
Peroxide ion	O_2^{2-}
Personal communication	*pers. comm*
Propylene glycol	PG

Q, R

Recrystallisation temperature	T_r
Reduced glutathione	GSH
Revolutions per. minute	rpm
Room temperature	RT

S

Scanning electron microscopy	SEM
Second	s.
90° side-scatter	SSC
Singlet oxygen	$^1O_2$
Sodium hydroxide	NaOH
Species	spp.
Sulfhydryl groups	SH
Superoxide dismutase	SOD
Superoxide radical	$O_2^{\cdot-}$

T

Thiobarbitauic acid	TBA
Thiobarbituric acid reactive substances	TBARS
Transmission electron microscopy	TEM
Trichloroacetic acid	TCA
Triplet oxygen	$^3O_2$

U

Ultraviolet	UV
United Kingdom Federation of Culture Collections	UKFCC

V

Version	v.
Video text overlay	VTO
Volume/volume	v/v

W

Weight/volume	w/v
Weight/weight	w/w
World Data Centre on Microorganisms	WDCM
World Federation for Culture Collections	WFCC

X,Y,Z

Chapter 1.

Introduction.

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1. Introduction

The primary goal of any conservation strategy, is the guarantee of present and future options (*e.g.*, for biotechnology, the reintroduction of endangered species and genetic enhancement) by sustaining biological diversity at genetic, species, population and ecosystem levels (Mangel *et al.,* 1996). Even with an organised conservation strategy the possibility remains that organisms may, eventually, be driven to a point at which, the genetic pool available to the organism will fall to a point at which it is no longer possible to maintain a healthy and reproductive population. If a species is defined as a potentially inter-breeding population, then the diversity existing in the gene pool is manifested as its resilience in response to selection, whether natural or artificial (Kresovich & McFerson, 1992). Consequently, a reduction in the diversity/size of an available gene pool will decrease the population fitness. Furthermore, the loss of natural habitats has made the maintenance of healthy populations of wild living resources increasingly difficult and it has been estimated that at the present rate of loss and destruction of natural environments, 25-50% of the species on earth will become extinct during the next 25 years (Myers, 1993).

Organisms, particularly vulnerable to extinction, be it on a local or global scale, are those with a limited distribution and which inhabit specific niche habitats; or those found in habitats which are subject to stress. Loss of diversity may occur as a consequence of environmental factors including: pollution, habitat loss, over exploitation, the introduction of non-native species, global changes and changes in land use. In response, strategies have been developed for the conservation of the earth's biodiversity, collectively presented in the *Convention on Biological Diversity* (Groombridge, 1992).

1.1 The importance of conserving genetic and biological diversity

Domestication of crop plants through traditional plant breeding involves the application of strong genetic selection pressures for desirable traits. Thus, the selective propagation of lines containing favourable traits has been accompanied by the progressive narrowing of the genetic base of subsequent populations (Tanksley & McCouch, 1997). Genetic

variation in crop plants has been further reduced by traditional plant breeding methodologies where new cultivars (as compared to their wild relatives) can be derived by breeding genetically related modern cultivars at the exclusion of genetically more diverse, but less productive, primitive ancestors. The consequence is plainly illustrated by the North American soybean, for which virtually all varieties can be traced back to a dozen strains from a small area of north eastern China. A further example is hard red winter wheat, where the majority of varieties originate from just two lines imported from Russia and Poland (Tanksley & McCouch, 1997). Biotechnological approaches to plant breeding have considerable potential, but they may also have important consequences for the diversity of natural populations.

Limited genetic diversity confers vulnerability to disease and insect epidemics and jeopardises the long-term potential for sustained genetic improvement (Tanksley & McCouch, 1997). This was highlighted in 1970 when corn yields in the United States of America were drastically reduced by an outbreak of Southern corn leaf blight, attributed to the extensive use of a single genetic male sterility factor that was genetically linked to disease susceptibility (Tanksley & McCouch, 1997). In response, recommendations were made that greater emphasis should be placed on collecting and preserving the genetic diversity of crop species with particular attention given to the conservation of the genetic diversity still present in remaining wild ancestors (Tanksley & McCouch, 1997). This addresses a primary requirement of plant breeding, which relies on sources of wide genetic variation to be used as the foundation of any effective crop improvement programme (Withers *et al.*, 1990), and thus germplasm repositories serve as convenient sources of genetic variation (Withers *et al.*, 1990).

With the advent of molecular mapping techniques it has become possible to scan the genomes of wild species for new and useful genes, allowing the exploitation of the wide repertoire of genetic variants created and selected by nature over hundreds of millions of years and retained in plant germplasm banks to be exploited (Tanksley & McCouch, 1997). Genetic engineering promises the possibility of modifying crop performance to enhance yields and resistance to pests. However, the value of collecting and maintaining the wild relatives of crop plants in gene banks, was predicted more than 50 years ago by Vavilov (1940). Global culture collections serve as reservoirs of microbes, cell lines and

as gene libraries. Their role is the supply of biological resources to biomedical, biotechnological and industrial communities (Ma *et al.*, 1995; Day & Turner, 1992).

1.2 Current status and level of conserving genetic resources

The importance of genetic diversity and the threat posed by the loss of this diversity to the world's agricultural food supply has already been recognised and this has lead to the development of *ex situ* and *in vitro* methods for the storage of germplasm. In addition, *ex situ* maintenance of germplasm forms the basis for the reintroduction of a diverse range of endangered species into areas where species have been eradicated by human pressures and for the enhancement of the genetic diversity in small populations (Herms *et al.*, 1996; Witkowski *et al.*, 1997; Bramwell, 1995; Nowell & Jackson, 1996).

1.2.1 *In situ* conservation

In situ conservation is preservation in the natural habitat and *in situ* maintenance of germplasm serves as a natural form of conservation with organisms fully exposed to natural evolutionary processes (Kochhar & Chandel, 1996). The main goals of genetic resource management are to acquire, maintain, distribute, characterise, regenerate, preserve, evaluate, and utilise the genetic diversity of crops and their wild relatives (Crossa *et al.*, 1994). Furthermore, these goals are equally important for non-crop plant genetic resources. The success of *in situ* conservation methods depends upon maintaining the distribution of genetic diversity from all the niches occupied by the organism. Assessments of the distribution of genetic diversity are paramount in devising conservation strategies (Brush *et al.*, 1995). *In situ* approaches for the conservation of crop germplasm in remote agricultural belts is possible because plants continue to breed where there is still a preponderance of local diverse germplasm and this has been suggested as a means of conserving rice crop species (Kochhar & Chandel, 1996).

Conservation of some fern species has been possible by utilising *in situ* spore banks (reservoirs in the soil of viable spores which remain dormant while buried but germinate in light if brought to the surface) and has allowed mature sporophytes of several British species to be raised (Dyer, 1994). However, in the majority of cases, conservation *in situ*

has been made almost impossible by the disappearance of large wild areas (Engelmann, 1991a). Furthermore, for those microorganisms which cannot, as yet, be cultured under *in vitro* conditions, *in situ* conservation in protected habitats is likely to remain the only viable option (Roper, 1993).

1.2.2 *Ex situ* conservation

Ex situ conservation is conservation outside the natural environment and includes field gene banks, seed banks and culture collections. Culture collections (repositories of genetic resources) are often maintained *in vitro* as non-dividing cryogenically stored material and/or as actively dividing cultures (*e.g.*, callus cultures of dedifferentiated cells or as differentiated cells).

Ex situ field management for the conservation of germplasm is both an economically and socially acceptable method (Singh, 1996). However, *ex situ* conservation in the field can be difficult to achieve because it requires that a representative genetic sample be first selected in order to conserve genetic diversity. Sampling varies from 20-30 plants for a single population to several hundred for gene pool conservation and commonly 5,000-20,000 plants for the maintenance of heterozygosity (Engelmann, 1991a). Thus, land space requirement is of primary importance. The conservation of forest genetic resources is further compounded by their immobility, longevity and the requirement for high genetic variation to be maintained as a long-term base for adaptability and survival (Behm *et al.*, 1997). In addition, many major crop plants are routinely propagated by seed, *e.g.*, rice, maize and wheat. However, there are a number of crops which require asexual propagation and their preservation demands the maintenance of clonal material by a means of vegetative propagation (Lizarraga *et al.*, 1989). Furthermore, it is required that virus and disease indexing of field maintained germplasm be performed in order to assess phyto-sanitary status. This requirement is necessary prior to international germplasm exchange. However, surveillance of the phyto-sanitary condition of organisms is costly and can be prohibitive in the establishment and functioning of germplasm collections (Withers & Williams, 1986). The clonal conservation of species in the field also requires planting, maintenance, harvesting and storage of material (Lizarraga *et al.*, 1989). To conserve species *ex situ*, therefore requires large areas of

land and it is labour and equipment intensive (Lizarraga *et al.*, 1989; Behm *et al.*, 1997). Moreover, plants maintained in field genebanks remain exposed to pests, pathogens, natural disasters, mismanagement, urban development and changing government policies (Engelmann, 1991a,b; Lizarraga *et al.*, 1989).

1.2.3 *In vitro* conservation

A more contained approach to *ex situ* conservation is the *in vitro* (*literally*, in glass) maintenance of genetic materials and germplasm in custom storage facilities, *e.g.*, at low temperatures, or in a desiccated state. *In vitro* culture of living material at slow growth rates, has also been adopted as a conservation method by botanical gardens around the world (Bramwell, 1995).

In recent years, the genetic conservation of 'problem crops' (*e.g.*, recalcitrant germplasm) which fail to respond to conventional long-term seed storage has attracted increasing attention from the International Plant Genetic Resources Institute (IPGRI). *In vitro* storage has been explored as a means of conserving the recalcitrant germplasm of these problem crop species (Withers *et al.*, 1990) and the method has been proposed as a safer alternative to the field bank (Withers, 1991a). Many species of plant may be maintained *in vitro*, as micropropagated plants or somatic embryos and are successfully employed for the conservation of potato and *Cassava* germplasm (Lizarraga *et al.*, 1989; Angel *et al.*, 1996). *In vitro* techniques are the principal maintenance method for microbial culture collections which secure microorganisms for exploitation and study by man (Kirsop & Doyle, 1991).

1.3 Importance of maintaining microorganisms

Microorganisms (*e.g.* viruses, bacteria, archaebacteria, protozoa, algae and fungi) are virtually ubiquitous and are interrelated with all other life forms, by being largely responsible for maintaining ecosystem function (Lipscomb, 1996; Wynn-Williams, 1996; Roper, 1993). They are extremely important in nutrient cycling mechanisms (*e.g.* nitrification of the soil and recycling dead organic material) and in the transformations of food, energy and chemicals, including the mineralisation of nutrients (Roper, 1993).

Their exploitation has also been proposed in novel applications including the use of efficient N_2 fixing rhizobia through the introduction of legume tree species to revegetate exposed subsoil in open mining areas and acidic residues resulting from bauxite mining in Brazil (Franco & De Faria, 1997). However, due to the present inability to successfully culture more than a few per cent of microorganisms, knowledge of their role and the range of their diversity is extremely limited (Roper, 1993).

Notwithstanding limited knowledge, it is essential to evaluate present and future threats to microbial diversity and attempt to minimise their impact. Microorganisms and microbial diversity risk being lost due to environmental factors such as pollution (by sewage, oil, organic compounds, pesticides and heavy metals); habitat loss due to a range of factors, (*e.g.*, pH and salinity changes, flooding); over exploitation (*e.g.*, in agriculture, forestry and fisheries); the introduction of exotic species; and through global environmental changes. Those microorganisms with a limited distribution which occupy specific habitats or those inhabiting niches which are subject to widespread stress are particularly vulnerable to extinction, be it on a local or global scale, (Roper, 1993). Loss of microbial diversity is accompanied by the potential for loss of ecosystem function because of their essential role in the maintenance of ecological systems.

1.4 The protists

The group of microorganisms collectively described as Protists include such diverse groups as eukaryotic algae and protozoa. These organisms, are only a few microns in size and may generally be described as being "plant like animals" or *vice versa*. They play wide ranging roles in the biosphere, and can be involved in many interactions with humans (*e.g.* by causing disease and infecting wounds).

The investigation of, and requirement to conserve protists, extends beyond their exploitation as useful organisms, as they may also have a deleterious impact on man. They are the causative agents of a number of diseases of humans and animals and are increasingly associated with newly emerging diseases caused by "opportunists" infecting immuno-compromised patients [e.g. transplant and human acquired immunodeficiency syndrome (AIDS) patients] (Beach *et al.*, 1997; Visvesvara *et al.*,

1997). For example, the algal infection of humans by *Prototheca* spp causing protothecosis, a cutaneous soft tissue infection, often poses diagnostic and therapeutic problems due to the rarity of the condition (Yang *et al.*, 1996; Gomez-Hernando *et al.*, 1996; Kim *et al.*, 1997). Increasing reports of *P. wickerhamii* have been associated with transient colonisation in cancer patients, lung transplant patients and AIDS patients, all of whom were immuno-compromised (Polk & Sanders 1997; Kwok & Schwartz, 1996; Gomez-Hernando *et al.*, 1996). Protistan infections also affect commercially important fish stocks including: herring, haddock and molluscs (Mellergaard, 1997; Bower *et al.*, 1997; Goggin & Lester, 1995; Chintala & Fisher, 1991). Similarly, protistan infections have been implicated in the widespread mortality of sea and eel grass beds which serve as important nurseries for many commercial fish species (Robblee *et al.*, 1991).

Algal "blooms" including red and brown tides are associated with reports of human disorders relating to the consumption of sea food contaminated by algal toxins (Premazzi & Volterra, 1993). Furthermore, toxins produced by the prokaryotic cyanobacteria (blue green algae) may lead to lethal poisonings, typically by haemorrhagic liver necrosis and they have caused mortalities in household pets and farm animals around the world (Codd *et al.*, 1995; Naegeli *et al.*, 1997). Biological toxins from macroalgae such as *Caulerpa* spp., or from benthic dinoflagellates such as *Gambierdiscus toxicus* may also act as principal stressors influencing reef fish health, by allowing opportunistic protistan parasites including amoebae to affect fish (Landsberg, 1995). These interactions between algae and other microorganisms have been implicated as the principle agent responsible for heavy mortalities in tropical reef fish (Landsberg, 1995).

1.5 The algae

Algae are a polyphyletic, artificial assemblage of O_2 evolving, photosynthetic organisms that include seaweeds (macroalgae) and an extraordinarily diverse group of microorganisms known as microalgae which embrace both prokaryotic and eukaryotic assemblages (Metting, 1996). The total number of algal species has been estimated at between one and ten million, with numbers mainly being composed of microalgae (Metting, 1996). They are ubiquitous in marine, freshwater and terrestrial habitats and

possess broad biochemical diversity. Their metabolic capabilities form the basis for present and future biotechnological and industrial applications through the exploitation of their capacity for producing complex biochemical compounds (Metting, 1996; Vandamme, 1992; Andersen, 1996).

1.5.1 Introduction to the algae

Algae have, in their broadest sense, existed on earth for the last 3.8 billion years (Bold & Wynne, 1985). Fossilised algal material dates from the Precambrian period (Bold & Wynne, 1985; Andersen, 1996). The oldest recognisable algae were prokaryotic and in many cases they are assumed to be similar to members of the cyanobacteria found today. Eukaryotic algae have been found in strata dating from as early as 1.2-1.4 billion years ago (Cloud *et al.*, 1969) and are considered to have been amongst the first classes of organism to begin the colonisation of earth. Higher plants with changed reproductive strategies and complex vegetative structure are not believed to have evolved until the Silurian/Devonian boundary (420-400 Ma), with the first direct fossil evidence for land animals only occurring in the late Silurian period (Edwards & Selden, 1992).

1.5.2 Evolution and distribution

The ability to evolve oxygen separates algae from protozoa and fungi (Stanier *et al.*, 1971). Distinguishing characteristics between the algae and other chlorophyllous plants resides in their mode of sexual reproduction. In unicellular algae the organisms themselves may function as gametes. In some multicellular algae the gametes may be produced in special unicellular gametangia. In others, the gametangia are multicellular, with every gametangial cell being fertile. None of these reproductive adaptations occur in liverworts, mosses or vascular plants, instead, the multicellular sex organs they possess are only partially fertile and covered by sterile cells (Bold & Wynne, 1985). In their asexual reproduction, many algae produce flagellate spores and/or non-motile spores in unicellular sporangia, or in multicellular sporangia, where every cell is fertile (Bold & Wynne, 1985).

Algae may be suspended in the water column (planktonic) or attached and living on a surface (benthic). A few algae are also neustonic, living at the interface between water and the atmosphere. They can be found throughout a wide expanse of salinity's, ranging from as low as 10ppm (NaCl) to marine waters of 33-40 ‰ (NaCl) to the Laguna Madre of Texas where the salinity may rise to 100 ‰ to mountain lakes of Virginia where the solute concentration is only 3.6 ‰ (Bold & Wynne, 1985). Algae can be found in the ice of either pole and to depths of 200m in clear tropical waters.

1.5.3 Classification

The algae have historically always included prokaryotic and eukaryotic forms (Smith, 1950), and comprise an assemblage of "plant like" organisms that are unified by their ability to photosynthesise. The one prokaryotic lineage of algae, the cyanobacteria (blue green algae) are more correctly classified as bacteria (Stanier *et al.*, 1971). However, these organisms continue to be considered as algae by many workers (*e.g.*, Bold & Wynne, 1985; Lee, 1989) and are retained in algal culture collections as opposed to bacterial collections (Andersen, 1996; Tompkins *et al.*, 1995).

Eukaryotic algae have, historically, been separated into three broad divisions: the Rhodophytes, the Chromophytes and the Chlorophytes. These major eukaryotic lineages are firmly established, but, relationships within these lineages are still under debate (Andersen, 1996; Cavalier-Smith, 1993). Furthermore, the classification of eukaryotic microorganisms (protists) has been in flux for over two centuries. Over the past 30 years the tendency has been to divide the protists into several kingdoms rather than to place them in one single kingdom as was proposed by Owen (kingdom protozoa, 1858), Hogg (kingdom Primignum, 1860) and Haeckel (kingdom Protista, 1866) (Cavalier-Smith, 1993). These earlier kingdoms included bacteria which were formally removed by Copeland in 1938 (Cavalier-Smith, 1993). Early attempts to subdivide protists simply into plants and animals, on the basis of their nutritional mode, photoautotrophy or heterotrophy, were abandoned because three well defined taxa (dinoflagellates, euglenoids and heterokonts) have some members of each type. Furthermore, many dinoflagellates and heterokont species are both photosynthetic and phagotrophic (Cavalier-Smith, 1993).

Recently, improved insights into protistan ultrastructure arising from electron microscopy have assisted in the proposition of specific phylogenies for protists. The increasing availability of molecular sequencing techniques has also been utilised as a valuable source of independent phylogenetic information (Cavalier-Smith, 1993). Revisions in the classification of algae are ongoing and this remains an area of conjecture, however, extensive future revisions will occur as new molecular data is accrued (Cavalier-Smith, 1993; Buchheim & Chapman, 1992; 1991; Buchheim *et al.*, 1990).

1.5.4 Algae as a valuable natural resource

Algae, account for approximately 50% of the total global photosynthetic activity (Wiessner *et al.,* 1995) and they form the basis of the food chain for 71% of the world's biomass (Andersen, 1996) with their biochemical diversity being exploited by applications in biotechnological and industrial fields (Metting, 1996). Algae are used as food products, as plant fertilisers, in cosmetics, in manufacturing processes and in biomedical research and "they find their way" into such everyday products as toothpaste, shoe polish, ice cream, lemonade and beer (Andersen, 1996; Wiessner, 1995). Industrial products from the algae include: agars, carrageenans and alginates (collectively the phycocolloids) and diatomite (fossilised diatom frustules). Within the context of medicine, examples of antibiotic activity, vermifuge activity, antitumor activity and goitre treatment have been reported (Stein & Borden, 1984; Cooper *et al.*, 1983). Extracts from seaweeds (macroalgae) sprayed on plants have been reported to reduce the incidence of *Botrytis cinerea* (gray mold) on strawberries, *Erysiphe polygoni* (powdery mildew) on turnips, and "damping off" of tomato seedlings (Kulik, 1995). In all, humans utilise approximately 500 species of alga as food or in food products and 160 species are considered to be of commercial importance (Abbott, 1988).

Microalgae are increasingly being utilised for the production of animal feed, human food, food additives, pharmaceuticals, and fine chemicals (Becker, 1994) (Table 1.1). Over the past decade, a series of reviews and books discussing cultivation of microalgae and the potential commercial applications of microalgal biotechnology have been

published, these include: Dixon *et al.*, 1997; Renn, 1997; Vilchez *et al.*, 1997; Borowitzka, 1996; Wilde & Benemann 1993; Duncan *et al.*, 1997; Johnson & Schroeder, 1995; Boussiba *et al.*, 1992.

Table 1.1 Examples of current major applications of microalgae

Alga	Use/Product	Status	Reference
Spirulina spp.	fish and shrimp feed	1	Benemann, 1992
Haematococcus spp.	astaxanthin	3	Johnson & An, 1991
Spirulina spp.	phycobiliproteins	3	Day & Turner, 1992
Dunaliella spp.	beta-carotene	3	Day & Turner, 1992
Chlorella spp.	health food	2	Day & Turner, 1992
Chlorella pyrenoidosa	ecotoxicity testing	2	Shubert, 1984
Chlorella vulgaris	ecotoxicity testing	2	Shubert, 1984
Selenastrum capricornutum	ecotoxicity testing	2	Shubert, 1984
	cryoalgotox	3	Benhra *et al.*,1997
Scenedesmus subspicatus	ecotoxicity testing	2	Shubert, 1984
Scenedesmus quadricauda	ecotoxicity testing	2	Day & Turner, 1992
Skeletonma costatum	ecotoxicity testing	2	Day & Turner, 1992
Ankistrodesmus braunii	ecotoxicity testing	2	Day & Turner, 1992
Asterionella spp.	ecotoxicity testing	2	Day & Turner, 1992
Euglena gracilis	ecotoxicity testing	2	Shubert, 1984
Ochromonas danica	biotin bioassy	2	Day & Turner, 1991
Amphidinium carteri	biotin bioassy	2	Day & Turner, 1991
Euglena gracilis	vitamin B_{12} bioassy	2	Day & Turner, 1992
Thallasiosira pseudonana	vitamin B_{12} bioassy	2	Day & Turner, 1992

1 Major commercial use
2 Commercial use
3 Small commercial use

At present, microalgae are used as feeds in aquaculture, mainly in the production of larvae and juvenile shell- and fin-fish, as well as for raising the zooplankton required for

feeding of juvenile animals (Benemann, 1992) (Table 1.1). Other current uses of microalgae are listed in Table 1.1.

Table 1.2 Examples of algal products under development

Algal product	Mode of action/application	Reference
Docosahexaenoic acid	reduced risk of heart disease	Conquer & Holub, 1996
Enzyme inhibitor	treatment of thrombosis, atherosclerosis, allergy and inflammation	Sekiya, 1997
Polysulfates	inhibit HIV[1,2] inhibit herpesvirus[2]	Ng *et al.*, 1997; Damonte, 1996
Biologically active compounds	inhibit bacteria and fungi that incite human diseases	Kulik, 1995; Stein & Borden, 1984; Cooper *et al.*, 1983
Biocontrol agents	inhibit plant pathogenic bacteria and fungi	Kulik, 1995
Porphyra products	lower blood cholesterol	Abe & Kaneda, 1972
Porphyra products	effective against stomach ulcers	Sakagami *et al.*, 1982
Porphyra products	reduced mammary cancer	Yamamoto & Maruyama, 1984
Porphyra products	lowered intestinal cancer	Yamamoto & Maruyama, 1985
Microalgae	vitamin production	Vandamme, 1992
Soil algae	ecological indicators	Shubert, 1992

[1]human immunodeficiency virus
[2] topical formulations to prevent sexual transmission

Future commercial applications include algal products with the ability to inhibit viruses. These are of great importance because the development of antiviral chemotherapy is

constrained by two main problems: (1) toxic side effects and (2) the emergence of drug resistance associated to the continuous treatment of immuno-compromised patients (Damonte, 1996; Ng *et al.*, 1997). Biomedical applications of algal products (Table 1.2), *e.g.*, polysulfates, have a great potential and can be obtained from natural sources such as marine algae and made available in large quantities at reasonable cost (Damonte, 1996). Further and future uses for microalgae include; disposal of animal waste by using animal waste as a substrate for protein synthesis (El-Ahraf & Willis, 1996) and for the development of microalgae high in lipids (omega-3 fatty acids) for use as substitutes for fish oil-based aquaculture feeds.

1.5.5 Astaxanthin: a case study

The carotenoids, of which astaxanthin is a member, form the most widely distributed class of pigments in nature. Their presence is essential to biological functions in animals, in addition, they impart an attractive pigmentation to many farmed and wild animals (Johnson & An, 1991). Animals lack the ability to synthesise carotenoids, which requires that farmed animals must be supplied with the pigments as supplements in their feeds. This is usually at considerable expense to the farmer and may contribute 10 to 15% of total feed costs (Johnson & An, 1991). Supplementing salmonid diets with astaxanthin rich microalgae, or synthetic pigments, results in significant deposition of carotenoids including astaxanthin, providing a visual enhanced flesh colouration of the salmonids (Sommer *et al.*, 1991). Due to the high cost of synthetic pigments there is a desire within the aquaculture industry to commercially exploit natural sources of astaxanthin (Johnson & An, 1991).

Many algal species are capable of astaxanthin production. These algae commonly encounter extremes of nutrient availability, acidity, solar irradiance, desiccation, and ambient temperature. Astaxanthin production has been associated with protecting the cell from some of these extremes (Bidigare *et al.*, 1993; Rise *et al.*, 1994; Yong & Lee, 1991). It is suggested that environmental or nutritional stresses, which interfere with cell division, trigger the accumulation of astaxanthin. (Boussiba & Vonshak, 1991). One example is the "snow alga" *Chlamydomonas nivalis* which accumulates secondary carotenoids in response to the lack of availability of nitrogenous nutrients (Bidigare *et*

al., 1993). This alga unusually includes large accumulations of astaxanthin esters in extra-chloroplastic lipid globules which produce a characteristic red pigmentation. These compounds greatly reduce the amount of light available for absorption by the light harvesting pigment-protein complexes, thus potentially limiting photoinhibition and photodamage caused by intense solar radiation (Bidigare *et al.*, 1993).

The green freshwater microalga *Haematococcus* is also commonly exposed to environmental extremes and it can produce high concentrations of astaxanthin (0.2 to 2% of biomass) (Cordero *et al.*, 1996). The accumulation of high levels of astaxanthin by *Haematococcus pluvialis* in response to extreme environmental conditions is a well recorded phenomenon (Harker *et al.*, 1996; Lotan & Hirschberg, 1995). Accumulation is associated with a change in the cell stage from biflagellate vegetative green cells to non-motile "reddish" aplanospore resting cells. The carotenoid accumulation has been reported under experimental conditions to occur in a few hours and without any morphological change in the algae (Chaumont & Thepenier, 1995).

Carotenoids appear to be efficient in protecting algal cells against photoinhibition and photooxidative damage if their content is greater than 1% of their dry biomass (Chaumont & Thepenier, 1995). An overall correlation between high cellular content of secondary carotenoids and the capacity to withstand excessive irradiation has been observed in flagellated cells and aplanospores of *H. lacustris*. (Hagen *et al.*, 1994). Furthermore, secondary carotenoids (astaxanthin, canthaxanthin and echinenone) of *H. lacustris* and *H. pluvialis* exhibit antioxidative activity which appears to provide protection from photooxidative damage (Hagen *et al.*, 1993a,b,c; Lotan & Hirschberg, 1995). Improved understanding of stress responses in the algae may assist in the development of successful cryopreservation protocols, *e.g.*, pre-acclimation of algae may induce biochemical changes which provide benefits during the cryopreservation protocol.

1.6 Culture collections, a conservation strategy

In recent years, the trend has not been towards conservation, *per se*, but rather towards sustainable utilisation and exploitation for national benefit (Hawksworth, 1996;

Hawksworth & Ritchie, 1993). Culture collections therefore satisfy a multitude of purposes in any conservation and sustainable exploitation strategy. They perform a service in support of biodiversity, as a tool for commercial sustainable exploitation (of the collections accessions), conservation (through the maintenance of accessions) and in restoration programmes (reintroduction from accessions). In addition, the collection and preservation of living specimens is essential for the elucidation of an organism's life history, through systematic and taxonomic investigation. Conserved specimens also allow the effect of human demands and climatic changes on biodiversity to be studied through the maintenance of accurate and verifiable taxonomic resources (Cotterill, 1995; Roper, 1993). Although understanding the relationship and evolutionary significance of allelic and quantitative genetic variation in collections and wild populations is still poorly understood, data on the fate of different types of genetic variation is required to allow the effectiveness of *ex situ* conservation to be evaluated (Hamilton, 1994).

Many culture and natural history collections exist throughout the world and retain a valuable source of genetic diversity in addition to that found *in situ*. They include zoos which act as *ex situ* collections of animals, botanical collections of *ex situ* plant species, numerous microbial and other specialist culture collections (*e.g.*, seed, tissue, egg and sperm banks).

Table 1.3 Culture collections affiliated to the World Federation for Culture Collections (WFCC)

Continent	Country	No.[1]	Continent	Country	No.[1]
Africa	Egypt	1	Europe	Austria	1
	Kenya	1		Belgium	5
	Nigeria	3		Bulgaria	3
	Senegal	2		Czech	15
	S. Africa	3		Denmark	2
	Uganda	1		Yugoslavia (former)	2
	Zimbabwe	2		Finland	2
				France	15
Asia	China	13		Germany	14
	India	12		Greece	4
	Indonesia	14		Hungary	6
	Iran	1		Ireland	2
	Israel	2		Italy	2
	Japan	23		Netherlands	8
	Jordan	1		NIS (former USSR)	10
	Korea (Rep.)	2		Norway	2
	Malaysia	3		Poland	5
	Pakistan	1		Portugal	1
	Philippines	8		Romania	1
	Singapore	2		Slovenia	1
	Sri Lanka	4		Spain	2
	Thailand	59		Sweden	4
	Turkey	2		Switzerland	1
				United Kingdom	25
N. America	Canada	28			
	Guatemala	1	Oceania	Australia	50
	Mexico	10		New Zealand	9
	USA	31		Papua New Guinea	1
S. America	Argentina	7			
	Brazil	44			
	Chile	1			
	Colombia	1			
	Venezuela	1			

[1] Number of culture collections per country.
Adapted from the World Data Centre on Microorganisms (WDCM, 1994).

1.6.1 Remit of culture collections

Culture collections are required to maintain their diverse range of organisms in a condition, representative of the original isolate, with, as far as possible, no change in the morphological or genetic form of the preserved material (Day & Turner, 1992; Grout, *et al.,* 1990; Snell, 1991). In rapidly dividing organisms the possibility of genetic change is likely to be noticeable, assuming a constant mutation rate per generation (Goodenough & Levine, 1975). Stability of material stored *in vitro* has now begun to be investigated. These studies include those of Angel *et al.* (1996) into variation at the molecular level in cassava plants after ten years *in vitro* storage. Genetic stability has also been reported for micropropagated meristems of potato (Potter & Jones, 1991), for tissue culture propagules of Pea leaflets (Rubluo *et al.*, 1984) and for meadow fescue plants (*Festuca pratensis*) regenerated from suspension cultures and protoplasts (Vallés *et al.*, 1993) However, genetic instabilities have been detected in potato maintained as callus cultures (Potter & Jones, 1991) and reports of variations in plant cell and tissue culture in other species have also been reported (Ting & Gu, 1990).

1.6.2 Microbial culture collections

Many of the examples listed above are representative of higher plant (*e.g.*, seed banking) and animal conservation (Table 1.3). In addition, to these higher organism conservation strategies there exist collections of microorganisms. Included within the Articles of the Convention on Biological Diversity is the principle of conservation and the study of microorganisms in their habitat (*in situ*) and their *ex situ* conservation in microbial culture collections (Kirsop, 1996). In microbial culture collections, conservation applies at three levels of biological diversity, the conservation of species, the conservation of genetic variation and the conservation of the environment. At present there are 484 culture collections, located in 58 different countries, registered with the World Federation of Culture Collections [World Data Centre on Microorganisms (WDCM) database] (Table 1.3). The World Federation for Culture Collections (WFCC) acts as a multidisciplinary commission concerned with the collection, authentication, maintenance and distribution of cultures of microorganisms and cultured cells. Their database includes information on > 810,000 individual microorganism cultures, held in

registered collections and the activities of 484 culture collections located in 58 countries (Ma *et al.*, 1995) (Table 1.3, Table 1.4). However, the difficulty in culturing many organisms under *in vitro* conditions imposes limitations on culture collections (Roper, 1993). It can therefore be assumed that the present level of cultures retained in collections does not truly represent the actual genetic diversity of the world.

Table 1.4 Diversity of microorganisms held in registered collections

Type of Microorganism	Number of cultures registered
Bacteria, including archaea	343,253
Filamentous fungi	372,304
Viruses	14,370
Cell lines	5,156
Other kinds of microorganism	80,485

Adapted from the World Data Centre on Microorganisms (WDCM, 1994).

Additional services provided by culture collections include: patent deposit services, confidential storage of strains for customers, distribution, identification, training and consultation. The stability of cells is of paramount importance in culture collections which serve as patent depositories under the terms of the Budapest treaty (Anonymous, 1995). The *ex situ* maintenance of algae may be a key factor allowing algae to be exploited as a source of natural products (Andersen, 1996). There are 66 algal culture collections registered with the WCDM data base. These include service collections and private laboratory collections and they are distributed around the world (Appendix I).

1.7 Maintenance methods used in culture collections

A number of maintenance methods have been adopted by higher organism and microorganism culture collections (1.7.1-1.7.2.5).

1.7.1 Serial subculture

The primary aim of a protistan collection is the preservation of viable protists in a state which, ideally, will arrest reproduction and so, selection. This will avoid the continuous selection which is intrinsic to a reproducing population and promotes deviation of biological characteristics, from those representative of the original population to those of a population adapted to culture conditions (Lumsden, 1972). Historically, protists have been maintained, *ex situ* by routine serial subculture (Pringsheim, 1946). This continues to be the method of choice for most phycologists, particularly when relatively small numbers of cultures are involved. However, this approach is suboptimal in service culture collections which act as repositories for cultures and are in effect gene banks of algal biodiversity. This is because the long-term maintenance of viable, healthy, stable cultures cannot be guaranteed by the collection. Furthermore, maintenance of the entire holdings of major collections by serial subculture requires significant resources (Day *et al.*, 1997). These factors have stimulated several of the largest protistan collections including the ATCC (McGrath *et al.*, 1978), CCAP (Morris, 1976a), NIES (Watanabe *et al.*, 1992) and the UTEX (Bodas *et al.*, 1995; Brand *et al.*, 1996) to develop methods suitable for the long-term preservation of their holdings.

As mentioned above, an important consideration in the conservation of germplasm is whether the germplasm in the gene bank, although initially stable, still represents at any point in time, the *in situ* populations from which it was collected, sometimes many decades ago (Del Rio *et al.*, 1997). Significant genetic differences have been detected between gene bank conserved and recollected *in situ* populations of potato germplasm. This has implications for the long-term maintenance of *ex situ* collections and may influence decisions regarding the value of recollection and *in situ* preservation (Del Rio *et al.*, 1997).

1.7.2 Alternative approaches to preservation

1.7.2.1 "Resting cells"

"Resting cell" formation in algae and cyanobacteria is a natural strategy, developed to withstand adverse environmental conditions (1.5.5). Examples include: *Haematococcus* spp. which produce aplanospores and many filamentous cyanobacteria, *e.g.*, *Anabaena* spp. which form akinetes. Some of these "resting cells" many survive for long periods, *e.g.*, cells of *Anabaena variabilis* and *Haematococcus lacustris* were reported as being able to germinate after extended dark preservation at 4°C (Braune, 1990).

1.7.2.2 Air Drying

In algae which develop resting stages, it is known that algal cysts/spores may be extremely resistant to desiccation and may survive prolonged exposure to dry conditions and high temperatures (Buzer *et al.*, 1985). This has permitted air dried soil samples containing *Haematococcus pluvialis* aplanospores to regenerate fresh cultures after 27 years storage (Leeson *et al.*, 1984). Drying has also been applied as a technique for the maintenance and preservation of fresh water unicellular green algae (Malik, 1993). Cultures of *Chlorella pyrenoidosa*, *Chlorella vulgaris*, *Selenastrum capricornutum* and *Scenedesmus subspicatus* have been liquid dried and cells proved viable and stable after reactivation (Malik, 1993). However, viability levels in stored material have been reported to drop during one year of subsequent storage at -30°C (Malik, 1993) or 4°C (Day *et al.*, 1987).

1.7.2.3 Lyophilisation

Lyophilisation has been successfully applied to the preservation of bacteria, viruses and pollen of many higher plant species (Heckly, 1985; Towill, 1985; Ashwood-Smith, 1980a). Attempts have been made to lyophilise unicellular green algae belonging to the *Chlorophyta* and *Chrysophyta*, however, only very low levels of post-thaw viability were obtained (McLellan *et al.*, 1991; McGrath *et al.*, 1978). Many protocols also

employ protective compounds, *e.g.*, sugars in a similar manor to cryoprotectants employed in cryopreservation protocols (1.8.5) (Franks, 1985).

1.7.2.4 Freezing and low temperature storage

Viable biological material may be maintained between 0°C and -100°C (Cromarty *et al.*, 1982; Pritchard, 1995). In the field of seed banking, the practise of storing orthodox seeds by desiccation, with maintenance at low temperatures, has allowed the long-term "banking" of seeds, under stable conditions, with a minimum labour requirement for the maintenance of the collection (Cromarty *et al.*, 1982; Pritchard, 1995).

In plant gene banks, seed storage at low temperatures and under dehydrated conditions, is a common practice. This technique is routinely applied for germplasm preservation in many botanical gardens. However, storage employing low temperatures and desiccation may only be applied to "orthodox" seeds. Seeds may be classified according to their tolerance to desiccation as: orthodox, intermediate or recalcitrant (Vazquez-Yanes & Arechiga,1996). Although few plants are capable of withstanding full desiccation, orthodox seeds undergo this event as a pre-programmed stage in their development (Kermode, 1997). In contrast, seeds from recalcitrant species do not acquire desiccation tolerance during their development and have relatively high water contents when they are shed from the parent plant (Kermode, 1997). These recalcitrant seeds cannot be stored for long periods of time at temperatures below 0°C because the high water content of the seeds may be converted into ice crystals. Intermediate seeds are unable to resist freezing temperatures and are only viable for a few years of storage at room temperature (Vazquez-Yanes & Arechiga,1996).

Recently, cryopreservation procedures have become more accessible for the long-term storage of plant germplasm. New cryopreservation techniques including: encapsulation/dehydration, vitrification and desiccation have extended the list of plant species which can tolerate low temperatures and, on recovery, retain normal growth (Villalobos & Engelmann, 1995). In addition, low temperature storage has also been successfully applied to fungal strains. In strains maintained at -80°C viability levels of 99.4% have been reported after 4 years of storage (Ito, 1991).

1.7.2.5 Cryopreservation

Cryopreservation involves the storage of viable cells, tissues and organisms at ultra-low temperatures, usually at the temperature of liquid phase nitrogen (-196°C) or in the vapour phase of nitrogen at -156°C. This preservation technique has been proposed as a method for the long-term maintenance of cells and tissues and there is increasing literature both on methodology (Day & McLellan, 1995a;b) and on fundamental aspects of cryopreservation (Grout & Morris, 1987; Steponkus, 1996; 1993; 1992). Cryopreservation has also been successfully applied to a number of biological systems, as a method for the long-term storage of viable reproductive material (*e.g.*, germplasm in sperm/egg and embryo banks) (Wildt *et al.*, 1997; Seal, 1996; Meyer *et al.*, 1996; Ademokun *et al.*, 1997; Kartha, 1985a,b; Hunter-Cevera & Belt, 1996). Storage at low and ultra-low temperatures has also been applied to the long-term stabilisation/preservation of microorganisms (Day & McLellan, 1995a; Lee & Soldo, 1992; Kirsop & Doyle, 1991).

1.8 Low temperature biology

The, first well documented experiment, demonstrating that the vivacity of an organism could be arrested by exposure to low temperatures was carried out by Henry Power in 1663 (Morris, 1981). The subsequent "resurrection" of eel worms after being frozen for several hours in vinegar indicated the possibility of preserving living material in a state of "suspended animation" (Morris, 1981). Further interest in the effects of low temperature was galvanised when, in 1683, Robert Boyle published *New Experiments and Observations Touching Cold*, a treatise which reported the possibility of using low temperatures to prevent decay in animal and plant tissues (Morris, 1981). In addition, the observations of Mantegazza in 1866 of the ability of human spermatozoa to survive freezing in semen at -17°C indicated that mammalian cells could be recovered after exposure to temperatures below their freezing point (Polge, 1980). Later observations by Jahnel in 1938 that a proportion of human spermatozoa could survive periods of storage in semen frozen to -80°C (solid CO_2) presented the possibility of using low temperatures for the long-term storage of biological material (Polge, 1980). Preservation

of material at ultra-low temperatures (-196°C) in liquid nitrogen (LN) (or in its vapour phase at approximately -150°C), by maintaining viable cells throughout the cryopreservation procedure, presented the possibility of routinely preserving material using low temperatures. However, following the early reports, little practical success was achieved in the application of low temperatures to the preservation of living material, with the exception of a few, naturally freeze tolerant forms.

From the seventeenth century until the 1940's, understanding of cryobiology remained largely unchanged. "*Life and Death at Low Temperatures*" (Luyet & Gehenio, 1940) compiled much of the information available before that time, with tentative hypotheses of the mechanisms involved in the preservation of life and of the causes of death at low temperatures (Luyet & Gehenio, 1940). Parkes revived interest in the cryopreservation of human spermatozoa in 1945 and a few years later, in 1949, one of the most important advances in cryobiology was made by Polge, Smith and Parkes. The discovery that glycerol provided a cryoprotective (antifreeze) benefit in avian spermatozoa, protecting fowl spermatozoa against freezing injury to a temperature of -80°C (Polge *et al.*, 1949). Major advances in low temperature biology and its application, followed with the development of a "general method" for freezing of animal cells (Smith, 1961). Today, cryopreservation is an important part of animal breeding programmes and mammalian cryopreservation methodologies are routinely applied in veterinary and healthcare fields including: transplant organ surgery, cell and tissue banking and human *in vitro*, fertilisation therapy (Watson, 1979; Karkow & Pegg, 1981; Testart *et al.*, 1986a,b; Curry, 1995; Hunter, 1995a,b; Palasz & Mapletoft, 1996; Denis *et al.*, 1996). Furthermore, fundamental and applied medical and veterinary fields have been able to benefit from the exploitation of cryopreserved cell lines retained by large commercial culture collections (Doyle *et al.*, 1988; Morris, 1995).

Developments in cryobiology since the formulation of a "general method" for freezing of animal cells by Smith (1961) have included an improved understanding of the modes of freezing injury and damage (Mazur, 1990a,b; Grout & Morris, 1987; Douzou, 1985; 1977; Steponkus, 1996; 1993; 1992; Kartha, 1985a; Ashwood-Smith & Farrant, 1980; Franks, 1985). Cryopreservation protocol development, has in recent years, included vitrification, with cells being rapidly cooled to allow the cells/cell suspension to achieve

a "glassy" vitreous state (Holt, 1997; Massip, 1996; Fahy *et al.*, 1987) and the use of alginate encapsulation techniques which ultimately permit the vitrification of the cells (Fabre & Dereuddre, 1990; Dereuddre *et al.*, 1992; Dereuddre, 1991; Janeiro *et al.*, 1996; Phunchindawan *et al.*, 1997; Mari *et al.*, 1995; Matsumoto & Sakai, 1995). Improvements in equipment have included the perfection of programmable freezers which allow complex cooling programmes and different cooling rates to be applied to the cryopreservation of cells (*e.g.*, the Planer Kryo 10 programmable freezers) and differential scanning calorimetry (DSC) which has allowed detailed investigations of the thermal histories of cryoprotectant solutions and permitted the optimisation and development of cryopreservation solutions and procedures (Ren *et al.*, 1994; Rubin *et al.*, 1990; Bronshteyn & Steponkus, 1993; 1992). Cryomicroscopy has also assisted the development of improved cryopreservation strategies and has furthered knowledge of cryobiology (Fleck *et al.*, 1997a; Chao *et al.*, 1996; Lee *et al.*, 1993; Morris *et al.*, 1988a,b; Mugnano *et al.*, 1995; Storey *et al.*, 1996; Scheiwe & Koerber, 1987).

In recent years low temperature biology has influenced many diverse fields of biology with interest currently being shown in the application of cryogenic methods for aiding the conservation of endangered species and valuable animal and plant groups (Maruyama *et al.*, 1997; Ballou, 1996; Touchell & Dixon, 1994; Touchell *et al.*, 1992). Cryopreservation has also increasingly been investigated as a stable, robust method for bringing "live" engineered tissue (both genetically engineered and synthetic tissues) products onto the market. Cryopreservation allows large stocks of these cells and tissues to be maintained (*in vitro*), ensuring a steady supply of material, to service the unpredictable demand for specific tissues in hospitals (Karlsson & Toner, 1996). In addition, the acquisition of a better understanding of the effects of temperature stresses on crops and the development of strategies to minimise frost damage, in particularly in agriculturally valuable species is of substantial economic importance (Larcher *et al.*, 1985). Furthermore, its continued use in artificial insemination for the breeding of cattle has significant commercial implications (Cseh *et al.*, 1996; Vidament *et al.*, 1997; Molinia & Rodger, 1996; Morrell, 1997).

The application of low temperature biology can extend the shelf life of products, by slowing or arresting microbiological spoiling of the food and has proven an effective

method for the extended storage of food (Reid, 1987). The use of much lower temperatures permits the storage of living organisms in a state of "suspended animation" for considerable periods and, with suitable cryopreservation protocols this approach is expected to yield viable cells for over 8000 years (Grout *et al.*, 1990). In addition, to the applications of cryobiology, where material is preserved for later use, low temperature biology has also lead to the development of cryosurgery, where ultra-low temperatures are used to kill damaged or infected cells including warts and cancers (Tatsutani *et al.*, 1996; Homasson *et al.*, 1994).

Plant cryobiology has also received considerable attention in recent years. This is primarily because of conservation and biotechnological interests in plant genetic diversity. A wide genepool is a prerequisite for crop improvement and germplasm is commonly retained in repositories of viable plant cultures (Withers *et al.*, 1990). Although the genetic resources of most crops can be conserved as seed, some vegetatively propagated crops and plants which produce recalcitrant seeds can not. *In vitro* storage methods utilising cryopreservation techniques are being developed to conserve such problem plants (Withers *et al.*, 1990). These cryopreservation techniques for plant cell cultures (*e.g.*, callus cultures, shoot cultures, embryo cultures) have been promoted as a key approach for the conservation of plants producing recalcitrant seeds (Withers, 1993; Withers, 1991a,b; Withers, 1989; Withers, 1985).

1.9 Cryopreservation

1.9.1 Low temperatures in natural environments

The responses of plants to environmental stresses, encountered, due to perturbations in their natural environment, particularly, wide extremes of temperature, have been of interest to man since early agriculturists began identifying "hardy" plants which survive environmental stresses and "tender" plants which do not (Levitt, 1980). As early as 1778, Bierkander reported that some plants were killed between 1°C and 2°C, and similar findings were reported by Göppert (1830) and Hardy (1844) (Levitt, 1980), prompting Molish to suggest in 1897 that low temperature damage in the absence of freezing should be called chilling injury as opposed to freezing injury (Levitt, 1980).

However, some plants can survive prolonged exposure to low temperatures (Hirsh, 1987); in addition, desmids have been reported to be able to survive in frozen sediment for in excess of 70 days (Nizam, 1960). Algae are also found associated with ice in the Antarctic and the unicellular planktonic algae *Dunaliella* spp., *Chlamydomonas* spp. and *Pyramimonas* spp. remain motile to temperatures as low as -10°C (Watanabe, 1988; Burch & Marchant, 1983).

Since the initial investigations into the effects of freezing temperatures and the role of natural cryoprotective agents in plants by Maximov (Levitt, 1980) understanding of the mechanisms of cold tolerance have greatly improved. Cold tolerant woody and herbaceous plant species exhibit freezing tolerance, or frost hardening, which can be induced by cold hardening during prolonged exposure of plants to low temperatures (Huner *et al.*, 1992). Freezing stresses and plant survival has attracted considerable interest in recent years with studies focusing on the freezing of plant cells, water and aqueous solutions, winter damage, frost damage, cold acclimation and freezing mechanisms (Sakai & Larcher, 1987). Cold hardy plants are generally assumed to survive freezing temperatures by avoiding intracellular freezing and tolerating extracellular freezing by deep supercooling of cytosol (Hirsh & Williams, 1985). *Populus balsamifera* and other woody plants from the far northern hemisphere can resist the stresses of freezing to below -28°C through the slow transformation of most of their intracellular contents to a glassy state (Hirsh *et al.*, 1985; Hirsh, 1987). Their ability to form stable intracellular glasses in hardy tissues, whilst in the presence of extracellular ice, has been linked to the presence of cellular sugars, *e.g.*, the trisaccharide raffinose and the tetrasaccharide stachyose (Hirsh, 1987). In addition, two further modes of avoiding low temperature stress have been identified in trees and shrubs. The first group exhibit deep supercooling and their distribution is limited to areas where the minimum temperature does not fall below the homogeneous ice nucleation temperature (Ashworth *et al.*, 1996; Goldstein *et al.*, 1985; Lipp *et al.*, 1994). The second group of woody species undergo extracellular freezing and their distribution is not temperature dependant but rather dependant upon the cells capacity to survive cryodehydration and the stresses accompanying intracellular ice formation (Ashworth *et al.*, 1996; Lipp *et al.*, 1994).

Antarctic organisms are also subjected to a harsh environment including extremes of light and temperature which influence and alter cellular life histories, physiology and anatomical characteristics (Wiencke, 1996); these include the seasonal production of antifreeze agents, initiated by environmental stimulants other than low temperature, *e.g.*, short day lengths (Johnston, 1990; Huner *et al.*, 1992). Ecological observations indicate that ice has a major effect on the occurrence and distribution of algae and ecophysiological studies have demonstrated that Antarctic macroalgae possess various adaptations to ice, low temperatures, and strongly seasonal light conditions (Clayton, 1994). Polar microalgae have been reported to adjust their lipid component, polyunsaturate contents and exhibit characteristic fatty acid decosahaenoic acid profiles as a strategy of adaptation to low temperatures (Huner *et al.*, 1992). The dimethylsulfoniopropionate (DMSP) content in the ice alga *Phaeocystis pouchetii* has been proposed to have a twofold biological role: as an osmolyte and/or as a cryoprotectant (antifreeze, agent) (Kirst, *et al.*, 1991). DMSP function has also been implicated in cryoprotection in the macroalgae *Ulothrix implexa*, *Ulothrix subflaccida*, *Enteromorpha bulbosa* and *Acrosiphonia arcta* from Antarctica and the subantarctic/cold temperate alga *Ulva rigida* (Karsten *et al.*, 1992). Cold tolerance has also been reported in *Zygnema* spp. (Chlorophyta) from the Antarctic, where intracellular ice nucleation could only be induced with high cooling rates (Hawes, 1990). However, *Zygnema* spp. could tolerate exposure to extracellular ice when slow cooling rates were employed and cells were able to maintain photosynthetic capacity with little loss of intracellular solutes after repeated overnight exposures to -4°C (Hawes, 1990). The Antarctic plant *Deschampsia antarctica* (Angiosperm: family Graminaceae), has also been reported to have unusually high accumulations of sucrose and fructans which are believed to act as cryoprotectants thus allowing it to grow at low ambient temperatures (Zuniga *et al.*, 1996).

Animals from polar seas also exhibit numerous adaptations to low temperatures, which prevent lethal freezing injury. Unique adaptations for freezing avoidance include the synthesis of low molecular mass ice-nucleating proteins, glycoproteins or peptides, that control and induce extracellular ice formation (Johnston, 1990). Marine poikilotherms also exhibit a range of adaptations which increase the rate of some physiological

processes, partially compensating for the effects of low temperature (Johnston, 1990). Some aspects of the physiology of polar marine species, such as low metabolism and slow growth rates, probably result from low temperature adaptations and the highly seasonal nature of food supplies (Johnston, 1990). In addition, wood frogs, can endure freezing for at least 2 weeks with up to 65% of their total body water in the form of ice (Storey *et al.*, 1996). Their adaptations to freezing include the capability to exhibit control over ice crystal growth in plasma with ice-nucleating proteins, the accumulation of low molecular weight cryoprotectants to minimise intracellular dehydration and the stabilisation of macromolecular components, and good ischemia tolerance by all organs (possibly including metabolic arrest mechanisms to reduce organ energy requirements whilst frozen) (Storey *et al.*, 1996; Storey, 1990).

Accumulation of the disaccharide trehalose has been observed in yeast, fungal spores, brine shrimp cysts and soil dwelling nematodes, and the accumulation the amino acid proline in haploid plants and marine invertebrates are examples of natural cryoprotectants (Rudolph & Crowe, 1985; Behm, 1997). The strategy of accumulating compounds which prevent deleterious alterations in membranes during reduced water states (a common point of cryoinjury) have also been successfully employed in the cryopreservation of tissues (Rudolph & Crowe, 1985; Madden *et al.*, 1993; Crowe *et al.*, 1985; Bhandal *et al*, 1985).

1.9.2 Temperature and the condensed phases of water

Changes in temperature can alter membrane electrical properties and because temperature changes are indicative of a freeze thaw cycle they must be considered in the development of any cryopreservation protocol, and temperature can be demonstrated to influence membrane electrical properties independently of cryoprotectants (Chekurova *et al.*, 1990). Exposure to solutions at lower temperatures (exposure temperature of 34°C & 0°C) have been demonstrated to reduce, by 18 fold, the lethality of solutions of epsilon-toxin (Lindsay, 1996). Reduced toxicity at lower temperatures may be due to the toxin interacting with cell surfaces via a temperature sensitive mechanism, *e.g.*, permeability. The possibility of reducing the toxicity of a solution by exposing cells to the solution at lower temperatures clearly has important implications in cryobiology.

This has been demonstrated in the cryopreservation of human skin with propane-1,2-diol where the use of low permeation temperatures (4°C) yielded higher post treatment viability using a controlled two-step cooling protocol (Villalba *et al.*, 1996). Further studies investigating the effect of temperature and incubation time on the toxicity of propane-1,2-diol to mouse zygotes demonstrated that toxicity acted in a temperature and time dependent fashion (Mahadevan & Miller, 1997).

After the cryoprotectant exposure step, during which cells may be exposed to potentially toxic solutions (at or above 0°C), the material for cryopreservation is further cooled. Cooling will commonly permit the intracellular and extracellular solutions to undergo some degree of supercooling with further reduction in temperature inducing phase changes. Cryopreserved biological materials are likely to encounter three distinct condensed solute phases: liquid, glassy (vitreous) or crystalline. The condensed (*i.e.*, water below 100°C) phases of the solution owe their existence to the interactions between the constituent particles: atoms, ions, or molecules. Each state has important consequences for the successful preservation of viable material.

Supercooling, observers in the early 19th century recognised that on cooling a liquid below its thermodynamic freezing point the onset of crystallisation was found to be highly unpredictable and sample dependant (Taylor, 1987). Although, the freezing point of cytoplasm is usually above -1°C, cells can, and generally do, remain unfrozen and therefore supercool to -10 or -15°C even when ice is present in the external medium (Mazur, 1970). The ability of cells to supercool even when external ice is present, indicates the absence of effective ice nucleators within the interior of the cells and the ability of the cell membrane to block nucleation (Mazur, 1970).

The ability of solutions to cool beyond the equilibrium freezing point (T_m) is governed by the fact that before crystallisation takes place, it must first be preceded by a nucleation event in which a solid-to-liquid interface is created (Taylor, 1987). The "nucleus" is a cluster of water molecules with a configuration which can be identified by other water molecules as "ice like", around which other molecules in the solution then condense (Taylor, 1987). In cooled solutions these "ice nuclei" formed by random motion occur spontaneously, however, they must exist for sufficient time to allow

crystal growth through condensation of further molecules. In a solution at 0°C the number of molecules necessary for nucleus formation is high, with the effect that the number of such nucleating clusters in a given sample of liquid is very low and their life time very short. However, with further cooling the number of molecules required to act as a critical nucleus decreases with the result that the number of nucleation points in a given sample increases.

Homogeneous ice nucleation is the spontaneous nucleation of ice by the above events and the absolute lowest temperature at which the liquid state exists is the homogeneous ice nucleation temperature (T_h). To form the first particle of the new phase a surface must be formed between the particle and the surrounding liquid. The creation of the surface requires energy, supplied by the release of energy resulting from the crystallisation process (MacFarlane, 1987). At, or just below the normal equilibrium melting point, the free energy associated with crystallisation is very small and hence the surface energy problem can only be solved by forming a very large particle (MacFarlane, 1987; Mehl, 1996a). The spontaneous occurrence of such a particle is highly improbable and hence the crystallisation is unlikely to occur until a much lower temperature where the free energy of crystallisation is larger (MacFarlane, 1987). The temperature at which the liquid begins to spontaneously form crystallites at a significant rate is termed the homogeneous nucleation temperature, (T_h) (Mazur, 1970; Toner, 1993; Stillinger, 1995). However, if suitable foreign surfaces are present, such that the interfacial tension between the new crystallite and the surface is lower, then crystal formation will occur preferentially on those sites and this is termed heterogeneous nucleation (Mehl, 1996a).

Heterogeneous ice nucleation is the process by which a liquid internally generates the first tiny crystallites of the new phase (MacFarlane, 1987). The presence of particulate matter and/or temperature gradients within a solution can provide interference and/or a template for ice formation allowing heterogeneous ice nucleation at temperatures above T_h. Ice formation in aqueous solutions is usually by heterogeneous nucleation, at temperatures well above the homogeneous ice nucleation temperature of water (-38.5°C) (Hobbs, 1974).

Furthermore, the apparent inability of membranes to block intracellular ice nucleation below -15°C has prompted the hypothesis that membranes contain water filled pores similar to those proposed by Solomon and others (Mazur, 1970). The barrier properties of membranes arise because at higher subzero temperatures (-10 to -20°C) ice crystals small enough to pass through such pores do not exist (Mazur, 1970; MacFarlane, 1987). Alternatively it has been hypothesised that intracellular ice formation may occur as a result of nucleation within the cell catalysed by either the plasma membrane or intracellular particles (Toner *et al.*, 1990). Recently, it has been hypothesised by Muldrew and McGann (1994) that the agent inducing intracellular ice formation may in fact be the osmotically driven water efflux which occurs during freezing (cryodehydration). This efflux of water is hypothesised to producing a rupture of the plasma membrane permitting the propagation of extracellular ice into the cytoplasm (Muldrew & McGann, 1994).

This recognition that biological membranes are not static, but have a liquid nature in their interior is important to cryopreservation (Franks, 1985; Mazur, 1970; Toner, 1993; Taylor, 1987). The multicomponent nature of cell membranes allows more than one phase to exist at any one time, *i.e.*, the system will not show a single sharp melting temperature since it is possible to vary the temperature of the system and still have both phases present (solid and liquid) (Taylor, 1987). This means that a membrane can gradually change its fluidity over a wide temperature range, allowing fine control of cell function determined by the temperature of its surroundings (Taylor, 1987).

Post nucleation: the liquid-solid phase transition is completed by the growth of ice nuclei into crystals of variable size and shape influenced by the extent of supercooling, rate of cooling, and the nature and concentration of dissolved solutes (Franks, 1985). The rate of nucleation rapidly increases with increasing cooling rate, with the opposite being true of ice crystal growth with slow cooling producing a small number of large crystals and rapid cooling producing a multitude of very small crystals (Taylor, 1987). Small ice crystals have high surface energies which render them thermodynamically less stable. Ultimately, unless a solution is frozen very slowly with a minimum of supercooling, metastable small ice crystals will form which will attempt to fuse, forming

larger, more stable crystals with lower surface energies (Franks, 1985). This process is encountered in the food industry where, even at constant storage temperatures, small ice crystals decrease in size while larger crystals grow (Donhowe & Hartel, 1996; Taylor, 1987). The number of crystals therefore decreases with time while their average size increases (Reid, 1983).

In the food industry the mechanism of recrystallisation and crystal growth have attracted a great deal of interest, particularly in the maintenance of ice cream quality through a series of freeze thaw cycles where recrystallisation will reduce product quality and shelf life (Donhowe & Hartel, 1996). Refrigerated ice cream is subjected to several recrystallisation mechanisms: melt-refreeze recrystallisation under oscillating-temperature conditions, rounding occurring at constant temperatures and migratory recrystallisation rarely occurred (Donhowe & Hartel, 1996). Recrystallisation has been reported to occur even with very small temperature fluctuations, with the crystallisation rate dependant upon the size of temperature fluctuation and storage temperature (Donhowe & Hartel, 1996). Storage temperature, amplitude of temperature fluctuation and period of fluctuation have all been demonstrated to influence ice crystal size and distribution (Donhowe & Hartel, 1996). These observations are applicable in the storage of cryopreserved material which may be stored for extended periods of time, presenting the possibility that a number of temperature fluctuations may be encountered. Although studies on ice cream were carried out at high subzero temperatures (-20°C), ice crystal growth and recrystallisation can be detected as low as -130°C (Taylor, 1987).

Vitrification, where cells are immobilised in an amorphous solid, presents the possibility of circumventing crystallisation. This is a potentially desirable attribute in the exploitation of low temperature systems and techniques (Franks, 1985). If T_h (the homogeneous nucleation temperature) is sufficiently low then it is possible that ice nucleation can be avoided, instead, as the liquid cools it becomes generally more viscous until it reaches a point where it can no longer flow on a measurable time scale (MacFarlane, 1987). The liquid now possesses the structural properties of a liquid but the mechanical properties of a solid and has become a glass (MacFarlane, 1987). The glass itself is commonly described in terms of viscosity [approximately 10^{14} N s m^{-2} or $>10^{13}$ poises (Franks, 1985; Grout *et al.* 1990)] and the glass transition viscosity

corresponds to a state in which there is effectively no diffusive movement of molecules on a measurable time scale (Franks, 1985). The phenomenon of glass formation presents itself in two important ways in cryobiology. Firstly, in cryopreservation protocols which utilise moderate levels of cryoprotectants and moderate cooling rates, where ice formation is inevitable, the "unfrozen" water fraction of these solutions vitrifies, resulting in a partially frozen solution (MacFarlane, 1987). Secondly, high concentrations of solutes may be employed, to attempt to avoid ice formation altogether by promoting the vitrification of the entire solution (MacFarlane, 1987; MacFarlane *et al.*, 1992; Steponkus *et al.*, 1992; Mehl, 1996a,b).

While in the glassy state, the vitrified material will relax towards its equilibrium state with time, dependant upon temperature, with the possibility of ice nucleation below the glass transition state (Mehl, 1996b). Fracturing in vitreous samples has been observed in many systems and has been suggested to be a cause of mortality (Hunt *et al.*, 1994; Pegg *et al.*, 1997). Fracture events are believed to be points of ice nucleation and crystal growth which cause irreversible mechanical damage within the cell. Fracture formation is believed to be intrinsic to the glassy state and is related to the ability of the glass to overcome thermal stresses within it. In practical vitrification solutions, ice nucleation may occur during cooling through heterogeneous processes, however, it is limited in highly concentrated vitrification solutions. During cooling, stable and unstable ice nuclei form and during storage, stabilisation of the unstable ice nuclei is possible.

As a glass relaxes, densification of the sample limits ice crystal growth, promoting storage at or just below the glass transition temperature (T_g) rather than at lower temperatures where fracture events are more likely (Mehl, 1996b). At, or close to, the glass transition temperature, the diffusion of water molecules will stabilise the ice nuclei, but prevent their growth. The homogeneous distribution of ice nuclei may also deplete the glassy matrix of excess water molecules limiting the crystal growth during subsequent warming (Mehl, 1996b).

Thawing can also have complex effects on cryopreserved material. The influence of warming rates and ice crystals on freeze fracture and devitrification events and the theories of vitrification/devitrification have been discussed in detail by Mehl (1996a,b),

Franks (1985), MacFarlane *et al.* (1992) and Stillinger (1995). By reducing the warming rate post-thaw function can be improved (Pegg *et al.*, 1997). In systems where fractures have been encountered and have probably resulted from thermal stresses created by rapid warming, fractures may be avoided by employing comparatively slow initial warming rates which allow the vitreous material to soften, reducing stresses and avoiding fractures, followed by rapid warming to ambient temperatures avoiding/limiting ice crystal nucleation/growth (Pegg *et al.*, 1997; Pegg *et al.*, 1996).

In specimens cooled under extreme non-equilibrium conditions, recrystallisation will occur during warming, as the very small ice crystals formed on cooling, which are thermodynamically metastable, minimise their surface to volume ratios by fusing or growing into larger, more stable ice forms. Although recrystallisation generally occurs at high subzero temperatures (T_r) it has been detected as low as -130°C (Toner, 1987). Changes in X-ray diffraction patterns in aqueous solutions correspond to the transformation of cubic ice to hexagonal ice, $I_c \rightarrow I_h$. It has been proposed that recrystallisation will occur along preferred axes so presenting diffraction's relating to different crystal shapes. In pure water, crystal growth rates along different axes have been demonstrated to vary substantially, dependant upon conditions such as supercooling and the presence of fish antifreeze glycoproteins MacFarlane *et al.*, 1992). There have been several types of recrystallisation identified each of which have been discussed in detail by Luyet (1965) (see below).

Irruptive recrystallisation: ephemeral spherulites formed during rapid cooling, resume their inhibited crystalline growth when the temperature reaches a specific narrow range (T_r) during slow rewarming. The event is easily visualised by the abrupt shift from a transparent preparation to an intensely opaque one (Luyet, 1965; Franks, 1985; Taylor, 1987).

Migratory recrystallisation: A gradual growth of large crystals in a population at the expense of the smaller ones. The migration of molecules from small to large crystals occurs as the specimen is warmed gradually from T_r to T_m, with the rate increasing with rising temperature (Luyet, 1965; Franks, 1985; Taylor, 1987).

Spontaneous recrystallisation: Can occur during rapid cooling, where the latent heat released during freezing, is able to cause localised temperature rises which can give rise to recrystallisation (Luyet, 1965; Franks, 1985; Taylor, 1987).

All recrystallisation occurs due to the highly unstable nature of solutions which have undergone rapid cooling. In these solutions the system will attempt, at a given temperature, to reduce its total surface area in order to reach its equilibrium state (the state achieved on slow cooling with initial freezing at T_r.

1.9.3 Cryopreservation

Cryopreservation, the storage of material at ultra-low temperatures, has been proposed as a method for the long-term maintenance of a wide range of biological material (Withers, 1987; James, 1987; Kartha, 1985a; Ashwood-Smith, 1980b; Polge, 1980; Whittingham, 1980; Kirsop & Snell, 1984; Morris, 1980; Fuller, 1987). Furthermore, it has been successfully employed in a number of biological systems as a method for the long-term storage of a wide range of viable material (Day & McLellan, 1995a; Lee & Soldo, 1992; Kirsop & Doyle, 1991).

The application of cooling to biological systems may have a number of extraordinary complex effects. These effects are primarily due to the temperature dependence of so many structures and processes. For example, cooling alters, to varying degrees, dissociation constants, retards chemical reactions and induces phase changes (notably in water and lipids) which can result in complex secondary effects (Franks, 1985). At temperatures used for cryopreservation (-196°C) normal cellular chemical reactions cease, as kinetic energy levels are too low to allow necessary molecular motion (Grout *et al.*, 1990). At -196°C the cell is effectively in an "arrested" state and theoretically the possibility exists for the long-term maintenance of material with little or no loss of viability. Studies on long-term cryopreserved algal cultures demonstrated no significant reduction in viability on up to 22 years of cryostorage (Day *et al.*, 1997). Some chemical damage can occur due to free radical formation and ionising radiation, possibly damaging nucleic acids and, in time, affecting genetic stability (Grout *et al.*, 1990).

However, despite a lack of enzymatic repair systems, irradiation due to background radiation would take 8800 years to reduce a population by 90% (Grout *et al.*, 1990). Cryopreservation, therefore, presents a tantalising opportunity for stable, long-term storage of biological material.

The earliest theories of freezing injury proposed that ice crystals formed in the extracellular solution, pierced or prised cells and intracellular structures apart, *i.e.*, via direct mechanical damage. It is generally true that intracellular freezing is lethal, however, the same cannot be said of extracellular freezing, as extracellular solutions are commonly seen to freeze during a two-step cryopreservation procedure. Osmotic stress, rather than ice crystals, is believed to be the direct cause of freezing injury to cells with cryoprotectant solutions, *e.g.*, containing glycerol acting to protect cells by modulating the rise in salt concentration during freezing (Polge *et al.*, 1949; Mazur, 1970; Pegg, 1987).

Most cryopreservation protocols are dependant upon cell dehydration and the concentration of the cytosol, in order to preclude or minimise ice crystallisation during freezing (Ashwood-Smith & Farrant, 1980; Farrant & Ashwood-Smith, 1980). The observation that intracellular ice formation was influenced by cooling rate was first made by Müller-Thurgau in 1886. More recently, the development of cryopreservation protocols which control concentration of the cytosol thus minimising injury and allowing viable cells to be obtained post-thaw have been developed (Day & McLellan, 1995b). During freezing, as ice forms in the extracellular solution, a gradient of water potential is established between the unfrozen intracellular space and the partially frozen extracellular compartment. The consequence is that the chemical potential of the water in the partially frozen extracellular solution decreases. If the cooling process is slow enough the cell will respond by loosing water across the semipermeable plasma membrane until the osmotic potential across the plasma membrane is returned to a state of equilibrium. In cells which are cooled too rapidly the chemical potential of the extracellular solution decreases much faster than the rate at which water can diffuse from the cell. The end result is intracellular ice formation in the cytosol (Franks, 1985; Farrant, 1980; Toner, 1993). However, freeze induced concentration of solutes is not a continuous function of temperature. The concentration of the remaining solution will

eventually reach a point at which it will form a glass so preventing any further concentration of the now supercooled cytosol. If sufficient concentration of the cytosol also occurs the cytosol will also vitrify. This will occur if sufficient time is allowed for osmotic equilibrium of the cytosol to occur, *i.e.*, in slowly cooled specimens (Franks, 1985; Farrant, 1980; Steponkus *et al.*, 1992). An optimal cooling rate exists because at too rapid cooling rates the probability of intracellular ice formation is increased, whereas at excessively slow cooling rates, the time the specimen is exposed to freeze concentrated solution is increased (Franks, 1985; Farrant, 1980; Steponkus *et al.*, 1992). Permeating cryoprotectant solutions are commonly introduced to mitigate injury encountered during freeze induced dehydration (Farrant, 1980; Steponkus *et al.*, 1992), *e.g.*, containing glycerol acting to protect cells by modulating the rise in salt concentration during freezing (Polge *et al.*, 1949).

1.9.4 Cryoprotectants

Successful cryopreservation of biological material normally depends upon the addition of exogenous cryoprotective compounds. The cryoprotectant solutions may be nominally divided into penetrating and non-penetrating compounds. Larger non-penetrating cryoprotectants are believed to act by protective dehydration of the cell, reducing the amount of cellular water available for intracellular ice formation (Kartha, 1985a,b; Farrant, 1980; Benson, 1990). The smaller penetrating, low molecular weight compounds are thought to protect the cell by lowering the temperature at which the intracellular water freezes (Kartha, 1985b; Franks, 1985; Benson, 1990). The solutes can, however, confer membrane protection by mechanisms other than by colligative action (action dependant on the concentration of chemical and not on its nature), by affecting membrane stability through specific solute membrane interactions, *e.g.*, reduction of the solute permeability of the membranes (Santarius, 1996). In the cryoprotection of thylakoid membranes by rapidly penetrating cryoprotectants the protection is predominately due to the colligative action of the solutes (Santarius, 1996). In systems with lower freezing temperatures the initial concentration of cryoprotectant must be higher to prevent the concentration of the potentially membrane-toxic solute in the unfrozen liquid from exceeding its critical limit (Santarius, 1996; Mazur, 1970).

This non-specific behaviour of the solutes explains the additive cryoprotective behaviour of cryoprotectants (Santarius, 1996).

At low temperatures insufficient concentrations of rapidly permeating cryoprotectants may act as cryosensitisers, accelerating membrane damage (Santarius, 1996). Under strongly hypertonic conditions, encountered during freezing, a gradual influx of non-penetrating solutes, including electrolytes, into the intrathylakoid space can take place. This may lead to membrane injury and the reports of increasing thylakoid damage with reductions in temperature in the presence of limited concentrations of permeating cryoprotectants may be explained by an increase in the membrane permeability to electrolytes (Santarius, 1996). During rapid freezing regimes, non-penetrating cryoprotectants have been demonstrated to be efficient in protecting thylakoid membranes (Santarius, 1996). The protective action of sugars is considered to be due to their ability to reduce the permeability of thylakoid membranes to solutes (Santarius, 1996; Hincha *et al.*, 1996). Sugars and carbohydrates have also been identified as being of importance in the overwinter survival of insects (Ushatinskaya, 1993). The cryoprotective efficiency of different solutes varies dramatically between different biological membrane systems and may indicate a complementary biochemical basis for cryoprotection. The naturally occurring cryoprotectants, trehalose and proline, have already found success in the cryopreservation of plant germplasm and mammalian tissue (Dalimata & Graham, 1997; Iwasaki *et al.*, 1995; Saha *et al.*, 1996; Benson, 1990). They may act to conserve membrane integrity during dehydration by the substitution of water molecules in membranes with sugar (Rudolf & Crowe, 1985). Cellular membranes have been previously identified as one of the primary sites of injury in frozen tissues and may therefore be protected through chemical stabilisation (Franks, 1985; Kartha, 1985b; Douzou, 1977; Benson, 1990).

In studies employing the cryoprotectants DMSO, glycerol and proline, maintenance of ATPase activity, coupling, unidirectional transport and membrane integrity were demonstrated in frozen and thawed endoplasmic reticulum (as reviewed by Benson, 1990). It has also been demonstrated that DMSO, ethylene glycol, and formamide decrease both the cell membrane permeability for ions and the membrane potential (Chekurova *et al.*, 1990). Evidence of non-colligative protective mechanisms of glycerol

exist in plants and animals (Benson, 1990; Piironen, 1993). In addition, there is evidence that cryoprotectants act as free radical scavengers (Benson, 1990; Benson & Withers, 1987). In studies where DMSO, a water miscible solvent, was used as the cryoprotectant evolution of methane has been detected indicating that DMSO was acting as a free radical scavenger (Benson & Withers, 1987). DMSO is also known to be able to induce cellular differentiation and act as a radioprotectant (as reviewed by Yu & Quinn, 1994). In addition, sugars are also able to act as free radical scavengers (Benson, 1990).

Cryoprotectants, which are essential for minimising cryoinjury during freezing, may be toxic to biological systems. Monohydric alcohols, dimethyl sulfoxide (DMSO), and ethylene glycol (EG) are known to denature enzymes at room temperature (Adam *et al.*, 1995). Furthermore, DMSO destabilises proteins at high temperatures, however, it has also been reported that DMSO may protect isolated enzymes during freezing (Adam *et al.*, 1995; Anchordoguy *et al.*, 1992). This apparent paradox was attributed to temperature dependent, hydrophobic interactions between DMSO and non-polar moieties of proteins (Arakawa *et al.*, 1990). In studies on the interaction of DMSO with phospholipid bilayers, leakage from phospholipid vesicles has been attributed to the destabilisation of phospholipid membranes due to a hydrophobic association between DMSO and the bilayer (Anchordoguy *et al.*, 1992). DMSO's action on the stability of the liquid matrix of cell membranes appears to be responsible; these actions also appear to cause related effects on membrane permeability and fusion (Yu & Quinn, 1994). In addition to the above, cryoprotectants such as DMSO, ethylene glycol, and formamide can decrease both cell membrane permeability for ions and the membrane potential with changes in membrane electric parameters depending upon the cryoprotectant type and concentration (Chekurova *et al.*, 1990).

1.9.5 Developing cryopreservation protocols

A common misconception is that a cryoprotocol successfully developed for one strain or species can then be readily applied to other similar cells or organisms. Although in some cases this has proven possible (Kartha, 1985a,b; Beaty & Parker, 1992), it is not generally the rule, with different microalgae requiring different cryoprotectants and

cooling rates to be employed (Fleck *et al.*, 1996; Day *et al.*, 1997). To successfully apply a cryobiological technique to another organisms often requires the development of a new cryopreservation protocol employing different cooling rates and/or cryoprotectants. Furthermore, culture age and life history stage may also influence the response of an organisms to cryopreservation (Canavate & Lubian, 1997a; Morris, 1981).

1.9.5.1 Controlled rate freezing

The development of cryopreservation techniques has been led by the use of two-step techniques, initially through the use of a cold immersion bath set at an intermediate temperature (*e.g.*, industrial methylated spirits (IMS) bath at -30°C) and, more recently, with the aid of programmable coolers (Day & McLellan, 1995b).

Two-step cryopreservation protocols commonly start with a period of exposure to a relatively high molarity cryoprotectant solution which a) concentrates the intracellular material through the removal, by osmotic action of intracellular water, and/or b) replaces a proportion of the intracellular water with a cryoprotectant solution. The cryopreservation procedures commonly use two-step "traditional" protocols. Either direct immersion in an IMS bath at a pre-selected intermediate temperature, prior to plunging in liquid nitrogen (LN, -196°C); or by controlled cooling in a programmable freezer, at a predetermined rate, to a selected intermediate temperature prior to plunging in LN. Each of these methods exploit the fact that chilling injury is dependent upon the rate of cooling (Grout *et al.*, 1990).

1.9.5.2 Vitrification

Vitrification: an approach to the cryopreservation of living material, which aims to prevent intracellular ice growth and limit or exclude the exposure of the material to the dehydration effects of extracellular ice is commonly achieved by adopting rapid cooling rates (Armitage, 1989). Vitrification strives to avoid/reduce the complex set of interacting variables which must be simultaneously optimised during two-step protocols, circumventing problems of chilling sensitivity/injury and removing the requirement for specialised expensive equipment. However, vitrification solutions remain prone to the

same problems of high osmotic potentials and toxic cryoprotective solutions encountered in two-step protocols. These difficulties are compounded by the highly concentrated solutions required for vitrification (Steponkus *et al.*, 1992).

Vitrification is readily achieved by cooling a viscous liquid below its thermodynamic freezing point, through its metastable supercooled regime, and finally below its "glass transition" temperature T*g* (Stillinger, 1995). The formation of a vitreous state is an intrinsic property of all liquids, and pure water and aqueous solutions will readily vitrify given a sufficiently high cooling rate (Macfarlane, 1987). Characteristically, vitrification solutions employ molecular liquids (*e.g.*, propylene glycol) at concentrations in excess of 40% (w/w) (Macfarlane, 1987). Vitrification solutions successfully developed for plant and animal systems are commonly comprised of high concentrations of conventional cryoprotectants (Armitage, 1986; Armitage & Rich, 1991) and/or more novel sugar constituents (sucrose) (Ishikawa *et al.*, 1997; Ishikawa *et al.*, 1996; Matsumoto *et al.*, 1997; Takagi *et al.*, 1997).

1.9.5.3 Encapsulation

Encapsulation has been used in numerous areas of medical and biological science including transplantation of encapsulated cells (Aebischer *et al.*, 1991a,b), dairy feeds (Ashes *et al.*, 1992), aquaculture vaccine delivery systems (Polk *et al.*, 1994) and plant tissue cryopreservation (Lee *et al.*, 1990; Fabre & Dereuddre, 1990). Increasingly, encapsulation is utilised as an approach for the oral administration of vaccines and drugs (Ellis, 1995; Morris *et al.*, 1994). The use of biodegradable polymer microspheres, or microcapsules, may have significant potential for adaptation to antigen release for immunisation. The encapsulation of antigens could act to prevent the acid and enzymatic degradation that has been a barrier to the development of oral vaccines (Morris *et al.*, 1994). It is also being investigated as treatment for diabetes, where the encapsulation of islets of Langerhans could be used, allowing insulin to permeate, into the body whilst protecting the transplanted cells from the hosts immune system (Goosen *et al.*, 1985; Zondervan *et al.*, 1991). This approach has also found uses in the wine industry, where yeast used in Champagne can be encapsulated to facilitate removal after

fermentation; it has also been used to retain better flavours and enhance the shelf life of spice and citrus flavourings in the food industry (Raghavan *et al.*, 1990).

Sodium alginate is used as the material of choice for the encapsulation of cells for cryopreservation. The affinity of alginate for metals permits cells to be first mixed with alginate before gelation, by ionotropic cross-linking gelation (or the linking of alginate molecules by cations, through exposure to a solution containing Ca^{2+} ions). At neutral pH, alginates provide anionic sites which bind ionically to metals but acidification releases metals when covalent bonds to H are formed (Crist *et al.*, 1994a,b). Sorbed heavy metals can be removed by precipitation of the sulfide (Cd), hydroxide (Cu, Al, and Ph), or as an EDTA complex (Pb) in ion exchange processes (Crist *et al.*, 1994a). This ability to bind metal ions may allow alginates to influence free radical production by removing metal ion catalysts which are involved in many free radical production pathways (see 1.11-1.12).

Encapsulation has proved to be a useful technique in cryobiology, allowing simple manipulation of encapsulated cells with sterile forceps, and the vitrification of materials without the use of high molarity, potentially toxic vitrification solutions. This technique has been employed with considerable success in plant tissue culture preservation (Fabre & Dereuddre, 1990; Dereuddre, 1992; Plessis *et al.*, 1993; Uragami *et al.*,1992; Scottez *et al.*, 1992; Bachiri *et al.*, 1995). Reports of successful application of encapsulation/dehydration to cryopreservation of: somatic embryos (Bachiri *et al.*, 1995), potato shoot tips (Bouafia *et al.*, 1996; Bouafia *et al.*, 1995), sugarcane apices (Gonzalezarnao *et al.*, 1996), *Dunaliella tertiolecta* a microalga (Hirata *et al.*, 1996), yam (Dioscorea spp.) shoot apices (Mandal *et al.*, 1996), shoot primordia *Vanda pumila* (Na & Kondo, 1996), *Solanum* shoot tips (Fabre & Dereuddre, 1990) and sugar beet shoot tips (Vandenbussche & Deproft, 1996). Encapsulation has also been used in the preservation of haemoglobin by freeze drying (Brandl & Gregoriadis, 1994) and in the standard cooling of rabbit embryos (Kojima *et al.*, 1990) and *Laminaria digitata* gametophytes (Vigneron *et al.*, 1997). In addition, horseradish hairy root cultures have been successfully cryopreserved by employing a hybrid form of encapsulation/dehydration and standard vitrification techniques (Phunchindawan *et al.*, 1997). In this study encapsulated cells were dehydrated using a highly concentrated

vitrification solution, prior to being plunged into liquid nitrogen, with good levels of post-thaw viability observed (Phunchindawan *et al.*, 1997).

1.9.5.4 Desiccation in higher plants

Conventional seed banks maintain orthodox seeds at about 5% (w/w) moisture content at -18°C. Using this method high viability levels can be maintained over many decades (Pritchard, 1995; Cromarty *et al.*, 1982). Cryopreservation has been advocated as a method which offers the possibility of much longer periods of seed storage. Preliminary indications suggest that the majority of orthodox seeds will respond favourably to cryopreservation (Pritchard, 1995). However, plant species which reproduce clonally fail to produce readily viable seed, or produce large fleshy seeds that are intolerant to drying to low moisture contents; alternative approaches including the cryopreservation of meristem, embryo and excised embryonic axis have been investigated (Pritchard, 1995; Benson, 1990). Although existing cryopreservation procedures have been employed the development of techniques which employ desiccation of somatic embryos are also emerging (Villalobos & Engelmann, 1995; Uragami *et al.*, 1992; Dereuddre, 1992; Uragami *et al.*, 1993). The use of a desiccation step, where somatic embryos are desiccated in a laminar flow bench of in the presence of slica gel has been successfully used to enhance post-thaw survival in the cryopreservation of oil palm (Dumet *et al.*, 1993a,b) and somatic embryos of *Camellia japonoca* (Janeiro *et al.*, 1996). In addition, high post-thaw viability levels have been attained where encapsulated plant tissues have been dehydrated employing a concentrated vitrification solution prior to plunging into LN (Phunchindawan *et al.*, 1997).

1.10 Stability of cryopreserved material

Cryopreservation at LN temperatures should permit the stable preservation of viable cellular material with no change in viability over extended periods of time (as measured in decades) (Day *et al.*, 1997). However, apparent changes in material stored using cryopreservation have been reported (Hajek *et al.*, 1995; Sakai; 1985). Reports of changes in post-thaw viability and responses of material after cryopreservation are of obvious concern to those who or wish to utilise this technique to preserve living

material. An alternative cause of changes in stored material could potentially be due to non-lethal chilling or freezing injury possibly as a result of free radical mediated events through the application of suboptimal cooling regimes (Patterson *et al.*, 1979; Serrano *et al.*, 1996; Omran, 1980). Also, thermal events associated with the normal day to day manipulation of a cryostore rather than events caused by cryopreservation itself could theoretically damage cryopreserved material. In the case of the apparent reduction in viability in *E. gracilis* the authors attributed the loss of cell viability to problems with the viability assays employed (Day *et al.*, 1997). The authors effectively discounted viability loss due to temperature perturbations during storage based on studies of the thermal stability of the cryostat and viability levels in other organisms maintained in the same cryostat (Day *et al.*, 1997).

1.11 Physical and biochemical damage

In frozen material, cell death is often indicative of intracellular ice formation, or the failure of chill injury protective mechanisms at a specific temperature resulting in mortality. The reduced survival of cryopreserved material can, however, also be attributed to a variety of additional factors. Expansion induced lysis, a consequence of large area deformations incurred by the plasma membrane during freezing and thawing of the suspending medium has been considered to cause "loss of function" in cryopreserved material (Meryman & Williams, 1985), with subsequent membrane rupture being linked to cell death (Morton-Firth *et al.*, 1996).

Many tropical and subtropical fruit and vegetables suffer chilling injuries when exposed to low temperatures and injuries often involve staining of the peel and internal browning, which can be related to modifications at the cell membrane level (Serrano *et al.*, 1996). Many of these deleterious effects have been attributed to free radical damage which may affect lipid bilayers, causing membrane deterioration (Serrano *et al.*, 1996). The importance of oxidative injury in crop species has led to the investigation of the application of transgenics to these plants with over expression of superoxide dismutase possibly providing increased protection from photooxidative damage (Allen *et al.*, 1997). In addition, higher plant thylakoids have also been demonstrated to be affected by temperature (Hincha *et al.*, 1996; Gilmore, 1997). Oxidative injury has also been

identified as a source of injury in mammalian tissues exposed to reduced temperatures (Bhaumik *et al.*, 1995; Deloecker *et al.*, 1997). In spermatozoa, membrane changes and damage have commonly been implicated in loss of function and viability after a freeze thaw cycle, even when the structural integrity of the spermatozoa was largely unaltered (Maxwell & Johnson, 1997; Henry *et al.*, 1993; Strom *et al.*, 1997; Valcarcel *et al.*, 1997).

1.11.1 Biochemical basis of cryoinjury

Membranes have been proposed as sites of freezing injury in plant cells (Benson, 1990; Santarius, 1990a,b; Lyons *et al.*, 1979). Injury has been observed as lysis of the cell or protoplast, leakage of electrolytes and other cell constituents, and the breakdown of fine structure (Singh & Miller, 1985). The concept of membrane reduction during cell contraction has been proposed as a mechanism of plasmolysis and extracellular freezing injury and was based on direct observations of reductions in cell volumes during plasmolysis (Williams *et al.*, 1981; Williams and Hope, 1981). In rye cells, there have been reports of irreversible conversion of the ultrastructural trilamellar membranous architecture to amorphous osmiophilic granules (Singh, 1981). Ultrastructural analysis of frozen-fixed rye cells during extracellular freezing, to lethal temperatures, have highlighted membranous responses in cells susceptible to injury, where membranes have been reported to roll up and fuse forming multilayered vesicles (Steponkus *et al.*, 1993). Eventually, the membrane bilayers may lose their phospholipid lamellar lattice altogether. There is further evidence from model liposome studies that the water of hydration around the phospholipid head groups is not only critical in the maintenance of the phospholipid lamellar lattice but is also instrumental in the prevention of fusion of adjacent bilayers (Singh & Miller, 1985). Perturbation of the water status in the vicinity of phospholipid bilayers by either dehydration or increased concentrations of intracellular cations (*e.g.*, Ca^{2+}), as encountered during extracellular freezing, may cause destabilisation of the bilayers leading to fusion (Singh & Miller, 1985). Although loss of functional bilayers may result in diminished membrane material and lysis on thawing (Wiest & Steponkus, 1978) damage occurring in the frozen environment cannot be precluded. In studies on artichoke tubers, losses of phosphatidylethanolamine and sterols from the plasma membranes and changes in high molecular weight proteins to

low molecular weight peptides have been attributed to degradation of freezing sensitive proteins by the activity of one or several proteases and other degradative enzymes (Uemura & Yoshida, 1986).

In support of theories where loss of compartmentalisation may be attributed to freezing injury, are series of reports describing degradation of phospholipids by phospholipase D (considered to be one of the lytic enzymes) together with evidence of its presence in a soluble form in plant cell vacuoles (Stout *et al.*, 1980; Rajashekar *et al.*, 1979, Yoshida, 1979a,b). These studies also demonstrate that lethal membrane alterations may occur during extracellular freezing releasing phospholipase D, degrading phospholipids and indicating that the disruption of membrane compartmentalisation, structure and function at lethal freezing temperatures may contribute to freeze recalcitrance (Singh & Miller, 1985). Alternatively, if the regulatory properties of membrane bound enzymes were impaired, degradation of membrane phospholipids may also occur (Yoshida, 1979a,b). Furthermore, in winter wheat varieties which are exposed to encasement in ice, an acclimation response which aids survival, has been observed as changes in enzyme activity (Andrews & Hope, 1994).

1.11.1.1 Evidence of biochemical cryoinjury in algae

In algae, large variation in resistance to cryopreservation has been observed between autrophically and heterotrophically grown cells and within given populations the effect of cell growth stage and age have also been shown to influence freeze tolerance (Morris *et al.*, 1979; Morris, 1976b; Morris & Canning, 1978). These reports point to the potential importance of biochemical changes and events within an organism in its ability to survive cryopreservation. However, in studies on the cryopreservation of *Tetraselmis* the preservation protocol employed had a greater influence on post-thaw viability than pre-culture under suboptimal conditions (Day & Fenwick, 1993; Fenwick & Day, 1992). Reports of leakage of cytoplasmic enzymes, including malate dehydrogenase and phosphatase, indicate increased permeability of the plasma membrane in *Euglena* and *Chlamydomonas* spp. (Morris & Canning, 1978; Grout *et al.*, 1980). However, the response of cell organelles to cryoprotectants may differ and may be influenced by their biochemical makeup, with different membranes being penetrated at different rates by

particular compounds (Finkle *et al.*, 1985). Tonoplasts have been reported to be more resistant to penetration by DMSO than plasma membranes (Delmer, 1979). This may be explained by the differences in membrane composition between the elastic membrane of the vacuole and the relatively rigid or fragile membrane of the protoplast (Neidermeyer, 1976).

In plant cells vacuole size is metabolically and nutritionally controlled, with decreased vacuole size having a corresponding increased lipid content (Finkle *et al.*, 1985). Studies have shown that shrinkage of vacuoles by osmotic dehydration may affect resistance to freezing (Pritchard *et al.*, 1986). Preculture of sycamore suspension cultures supplemented with 6% mannitol increased the resistance of cells to freezing, studies showed that preculture in the presence of mannitol or sorbitol caused a proportion of the cells to shrink and display structural alterations including an decrease in cell wall thickness and an appearance of being more densely cytoplasmic. In addition, cells osmotically stressed, showed a decrease in the cross sectional area of vacuoles (Pritchard *et al.*, 1986). Vacuole size has also been associated with freezing sensitivity in *Chlorella* spp., where a loss of vacuole integrity was seen to be a primary effect of freezing injury (Morris & Clarke, 1978). This presents the possibility of the activation/release of soluble phospholipases from the vacuole during freezing injury in *Chlorella* spp. (Morris & Clarke, 1978).

1.11.1.2 Further effects of temperature on biochemical processes

Additional effects of temperature on cellular biochemical processes are demonstrated by the ability of the temperature of cryoprotectant addition to mediate the effects of the cryoprotectant (Steponkus *et al.*, 1990) (Section 1.9.2). Observed benefits may be due to changes in the physical chemical characteristics of the aqueous cryoprotectant solution and/or the cells at different temperatures (Finkle *et al.*, 1985). Changes in the molecular arrangement in lipids at 15°C and 28°C in both mitochondrial and chloroplast membranes caused by phase transitions, indicate a variation in the fluidity of the membrane and hence its elasticity (Neidermeyer *et al.*, 1976). Ultrastructural differences between prechilled cells and room temperature cells have been detected after exposure

to cryoprotectants and have been attributed to differences in the degree of gelation of the membranes at the two temperatures (Finkle *et al.*, 1985).

In some protocols, cells may be cooled prior to the addition of cryoprotectants. The effects on cell structure after brief exposure to reduced temperatures include cells undergoing reversible as well as irreversible structural changes (Patterson *et al.*, 1979; Niki *et al.*, 1979). These changes include vesiculation of the endoplasmic reticulum, observation of free ribosomes (disassociation of ribosomes), increase in plastid density and disruption of mitochondrial cristae after periods of exposure at 0°C (Finkle *et al.*, 1985). Studies suggest that organelles of different species behave differently and that cell responses may be rapid (Finkle *et al.*, 1985). Furthermore, organelles within the same species are not equally affected and changes may be reversible under some conditions (Finkle *et al.*, 1985). Loss of cell turgor and distortion of the plasma membrane in the alga *Dunaliella* due to dehydration, proceeds an increase in the concentration of tissue abscisic acid, which has been correlated with induction of stress related gene expression. (Cowan *et al.*, 1997). This effect of dehydration on the expression of genes may be directly implicated in the stresses commonly encountered during cryopreservation.

Treatment of *Digitalis lanata* cells and protoplasts with osmotic agents and slow cooling (-0.5°C $min.^{-1}$) to -60°C in an osmotically active cryoprotectant solution resulted in considerable reduction of volume and surface of the protoplasts (Diettrich *et al.*, 1991). Both processes were accompanied with an increase in the number of membrane coated vesicles in the cytoplasm (Diettrich *et al.*, 1991). The observed shrinkage of the plasma membrane seemed to be brought about by two processes: endocytotic vesicle formation and extrusion of lipids that were deposited on the membrane (Diettrich *et al.*, 1991). In addition, cryoprotectants such as DMSO, ethylene glycol, and formamide may biochemically alter cells by decreasing both the cell membrane permeability for ions and the membrane potential (Chekurova *et al.*, 1990; Hincha *et al.*, 1996).

Many of the changes reported in senescing plant cells have been associated with modification of membrane properties, including alterations of the permeability of the

cell membrane to ions and metabolites (Merzlyak & Hendry, 1994). In leaves and ripening fruit, senescence is accompanied by a progressive decrease in chlorophyll content that is indicative of the degradation of the photosynthetic apparatus (Merzlyak & Hendry, 1994). In these and other senescent events, it has been assumed that there is a single basic cause of ageing, modified by genetic and environmental factors, with the postulation of free radical involvement (Merzlyak & Hendry, 1994). The reported involvement of free radicals in changes in membrane function may be of relevance in cryopreserved tissues, where, of all the manifestation of freezing stress, membrane damage has been one of the best documented (Benson, 1990).

1.11.2 Free radicals

Free radicals are a natural and intrinsic part of metabolic reactions in all living cells and one of their primary sources is oxygen. Oxygen, although not essential to all life on earth (many microorganisms are able to function anaerobically) is essential to the majority, including all higher and more complex organisms. In addition, oxygen is present at high levels in plants as a result of photosynthetic activity with semiquinone free radical intermediates involved in both photosystems I and II. Furthermore, due to the involvement of oxygen-derived free radicals in plant metabolic processes it is possible that the free radical content of metabolically active tissue may greatly exceed levels generated as a result of senescence processes and other forms of oxidative damage (Magill *et al.*, 1994). Free radicals may also be involved in essential growth and polymerisation reactions and may function as intercellular messengers.

Although oxygen is effectively ubiquitous in nature, at elevated levels it may be toxic. Oxygen toxicity is primarily attributed to free radicals derived from oxygen during cellular metabolism. However, they may also have an exogenous origin (Pierre, 1995). In general, major contributions of the total cellular production of oxygen metabolites come from membrane-bound enzymes. During aerobic respiration, powerful reducing agents are utilised (NADPH, NADH) and elaborate electron transfer chains are employed for the reduction of molecular oxygen to water (Nivière & Fontecave, 1995). Reduction of dioxygen (O_2) to H_2O occurs during cellular respiration in the

mitochondria. The reduction of each molecule of oxygen can be catalysed by cytochrome oxidase and other oxidases (Pierre, 1995).

Oxygen may exist in a number of reactive states, the non-toxic molecular triplet oxygen ($^3O_2$) molecule and its more reactive forms. Singlet oxygen ($^1O_2$, an excited and more reactive state of oxygen) can be formed via photosensitised reactions or by chemical excitation reactions, possibly by a radical-radical interaction. $^1O_2$ diffuses across membranes and can target membrane components, nucleic acids and proteins. However, it can be deactivated by a number of cellular compounds, including β-carotene (Pierre, 1995; Nivière & Fontecave, 1995).

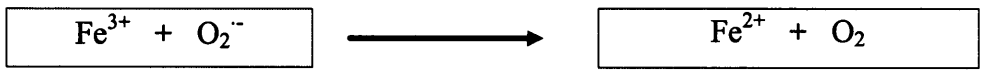
Photosynthetic organisms also have free radical formation associated with their photosynthetic cycles and therefore suffer from a further source of radical mediated damage. The high levels of free radical production, as a by-product of photosynthesis, are reflected in chloroplasts being the richest source of antioxidants (which act to limit the effects of excessive free radical activity by "mopping up" surplus radicals and deactivating them). In all photoautotrophic organisms O_2 evolution due to the Hill Reaction occurs and may promote production of potentially destructive free radical species. One of the primary reasons for the toxicity of free radicals is their highly reactive nature which promotes the production of cascades of damaging chain reactions in living plant tissue (Benson & Withers, 1987; Benson *et al.*, 1992a,b; Benson & Roubelakis-Angelakis, 1994; Benson & Noronha-Dutra, 1988).

In many biological reactions there is a close relationship between free radicals and transition metal ions which are involved in both radical generation and scavenging, *e.g.*, the dismutation of superoxide. In the presence of a Fe^{2+} catalyst the Haber-Weiss and Fenton reactions play an important role in producing the (highly toxic) hydroxyl radical $^{\cdot}OH$ from hydrogen peroxide (see below). The hydroxyl radicals may attack lipids, DNA, sugars etc. causing a wide variety of damage. The superoxide anion radical $O^{\cdot}_2{}^-$ (a strong reducing agent) can react to produce perhydroxyl, which through a series of

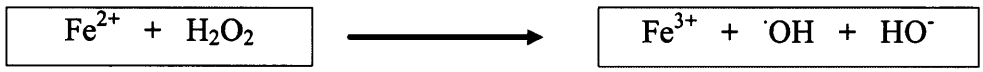
further reactions can form HO_2^- and on reaction with C-H may induce lipid peroxidation.

Production of hydroxyl radicals may be promoted via the Fenton and the Haber-Weiss reaction pathways (reviewed by Benson 1990). Haber proposed a reaction between $2O_2^{\cdot-}$ and H_2O_2 which is detailed below.

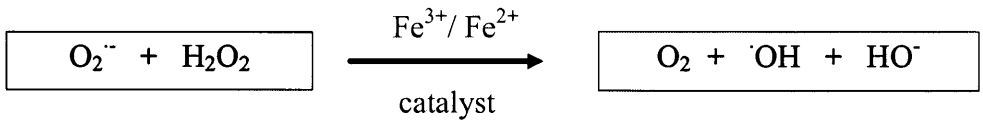
This original reaction was later found to proceed via two steps known as the Fenton reaction. Firstly the superoxide radical reduces the ferric ion:



Secondly, this interacts with H_2O_2 to produce the hydroxyl radical.



This is the major route for the formation of the highly reactive hydroxyl ($^{\cdot}OH$) radical in biological systems and may be summarised as:



In response to radical production plants produce a number of highly specialised metabolites (*e.g.*, phenols) as protective agents against oxidative damage. Antioxidant systems include enzymes and peptides, *e.g.*, superoxide dismutase (SOD) which catalyses the disproportionation of superoxide into hydrogen peroxide and oxygen. In

addition, catalase and peroxidases promote the destruction of some organic hydroperoxides and hydrogen peroxide, a precursor of $^{\cdot}OH$. Both SOD and catalase have been suggested as having a role in membrane protection (Dhindsa *et al.*, 1981) (See section 1.9.5).

The glutathione reductase enzyme reduces oxidised glutathione (GSSG) back to reduced glutathione (GSH) and is the same enzyme that catalyses the transfer of electrons from NADPH. GSH can react with many radicals non-enzymatically by donating an electron to form GSSG. Furthermore, antioxidants within cells also include the carotenoids which are effective free radical scavengers which are able to inhibit the formation of $^1O_2$ (Goodman, 1994). Plants have an enormous capacity for antioxidant protection and this is used to good effect in medicine. For example, the antioxidant function represents the central principle of the chemopreventive activity of carotenoids against cancer initiation and promotion (Dhindsa *et al.*, 1981). Further evidence for the antioxidant action of carotenoids is in copper-mediated lipid peroxidation in suspensions of *Haematococcus* cells with different amounts of secondary carotenoids where higher resistance to photooxidative stress was observed in cells which contained higher levels of secondary carotenoids (Hagen *et al.*, 1993b). Vitamin C (ascorbic acid) is also a well documented antioxidant and has been demonstrated to act in synergism with vitamin E (Halliwell & Gutteridge, 1986; Niki *et al.*, 1983; Benson, 1990).

Free radicals, and in particular those derived from oxygen, are also associated with plant responses to many external stresses including: dehydration, temperature extremes, heavy metals, herbicides, ionising radiation disease and senescence. Although the free radical responses detailed above are associated with environmental stresses, plants also encounter stresses during cryopreservation. In plants, free radical production and injury due to environmental stresses encountered throughout their evolution, *e.g.*, drought, flooding, frost, *etc.* has promoted the development of complex mechanisms for the control of free radical activity (antioxidants) (1.11.3). It may therefore be expected that in cryopreserved plant tissue, exposed to stresses during cryopreservation, *e.g.*, cryodehydration, use of high molarity cryoprotectant solutions and temperature extremes (-196°C) the same antioxidants may play a valuable role in overcoming cryoinjury.

1.11.3 Case studies on oxidative stress and protection by antioxidants

In studies comparing diet in mice, those mice fed diets without vitamin E (with or without supplemental iron) had significantly higher hepatic levels of thiobarbituric acid reactive substances (TBARS) (Ibrahim *et al.*, 1997). Levels of TBARS could be further increased by supplementation with iron (Ibrahim *et al.*, 1997). These increased levels of thiobarbituric acid reactive substances may be attributed to increased free radical activity causing lipid peroxidation. Lipid peroxidation has been implied as being an important cause of cell membrane damage through the degradation of polyunsaturated fatty acids of cell membranes with consequent disruption of membrane integrity (Yoshikawa *et al.*, 1997). It was suggested by Ibrahim (1997) that vitamin E protects lipid soluble compounds, and water soluble constituents against oxidative damage (Ibrahim *et al.*, 1997). In mice, fed fish oil and vitamin E, significantly higher levels of glutathione (GSH) were found in the liver than in those fed lard and vitamin E (Ibrahim *et al.*, 1997). The activities of superoxide dismutase and glucose-6-phosphate dehydrogenase were also lower in the fish oil fed mice than in those fed lard (Ibrahim *et al.*, 1997).

Further examples of the importance of antioxidants in the diet come from less well developed countries where imbalances between antioxidant protection and pro-oxidant stress have been reported to accurately predict survival in malnourished children (Houssaini *et al.*, 1997). Studies also suggests that dietary intake of olive oil polyphenols may lower the risk of reactive oxygen metabolite mediated diseases such as some gastrointestinal diseases and atherosclerosis (Manna *et al.*, 1997). There have also been many demonstrations in animals that natural antioxidant compounds, including flavanoids, diminish or prevent both acute and chronic pathologic changes related to treatment with anthracycline antibiotics (a drug used for treatment of hematopoietic malignancies and advanced solid malignant tumours) (Bogdanovic *et al.*, 1996). Furthermore, the Japanese and Chinese crude drugs (Kampo drugs) have also been found to possess antioxidant activity (Ogata *et al.*, 1997). Their antioxidant activities differ in that those containing phenolic compounds with allyl groups were effective antioxidants because of their scavenging ability for superoxide or hydroxyl radicals,

whereas other phenols, without an allyl moiety appeared to play a role in the termination of free radical chain reactions (Ogata *et al.*, 1997). In addition, oxidative stress has also been considered to be of importance in aquatic animals, with temperature stress causing changes in SOD and GSH-Px activities and GSH (thiol) content in fish gills (Parihar *et al.*, 1997).

1.11.4 Free radical damage and cryoinjury

Free radical damage has been implicated in a number of mammalian disorders including cancer, DNA damage and radiation injury, prompting interest in free radicals and their interactions with tissues. It has also been hypothesised that free radical mediated effects may also influence plant genetic stability *in vitro* and in stored seed (Benson, 1990).

Studies on free radical activity in plant and animal systems have prompted the investigation of free radical damage induced during cryopreservation of plant and animal tissues. Already, a number of examples of free radical mediated effects in plants have been documented (Crawford *et al.*, 1994). In cryopreserved plant germplasm the importance of free radical activity has yet to be fully substantiated. However, in mammalian tissues stored at low and ultra-low temperatures free radical toxicity has increasingly been reported as an important source of injury (Fuller *et al.*, 1988; Fuller 1988; Cotterill *et al.*, 1989a,b; Green *et al.*, 1986a,b; Gower *et al.*, 1989a; Fuller *et al.*, 1985; 1996). This is demonstrated by the use of exogenous antioxidants to stabilise tissues at low temperatures (Benson, 1990). In low temperature stored seeds (*ca.*, -18°C) studies have already demonstrated free radical damage and its importance in seed deterioration (Benson, 1990).

Free radical activity at temperatures below 0°C has also been observed. In the food industry, where low temperature storage is commonly used to extend a product's shelf life (-12°C - 18°C), loss of membrane integrity, largely due to oxidative stress, has been reported in lobster and in frozen fish. In frozen fish, lipid break down was believed to be accelerated by ice crystal formation (Benson, 1990). Similarly lipid oxidation has been reported to be one of the major mechanisms of the deterioration of frozen meat (Benson, 1990). At the lower temperatures used in the storage of heart muscle, free radical

activity has been demonstrated during freezing and thawing of tissues held at -40°C and between -70°C and -80°C (Lange *et al.*, 1980).

Free radical mediated damage has been further implicated in the physiological deterioration of organs following low temperature storage (Janjic *et al.*, 1996; Schon *et al.*, 1993; Fuller *et al.*, 1988; Fuller, 1988; Cotterill *et al.*, 1989a,b; Gower *et al.*, 1989a; Green *et al.*, 1986a). In addition, the series of metabolic events which predispose transplant tissue to free radical damage have also been observed in plant tissue similarly exposed to low temperatures, these include mitochondrial arrest, loss of cytochrome C and membrane damage (Benson, 1990). Studies have indicated that in *Daucus carota* free radical mediated lipid peroxidation can occur during early post-thaw recovery (Benson & Noronha-Dutra, 1988). Comparable studies on cryopreserved shoot tips of *Brassica napus* have linked $^1O_2$ to early post-thaw injury (Benson & Noronha-Dutra, 1988). These examples of free radical mediated damage in plant systems (Benson *et al.*, 1992a,b) are supported by parallel studies in animal systems which have shown low temperature stress to be mediated by oxidative damage (Green *et al.*, 1986a,b). Although free radical activity has been evidenced through the detection of lipid peroxidation products in tissue specimens stored at high subzero temperatures (-20°C and -70°C) no evidence of lipid peroxidation or free radical activity has been shown at -196°C. This finding can be considered to be further evidence for the suitability of employing ultra-low temperatures for the long-term storage of biological material (Whiteley *et al.*, 1992a,b).

In many cryopreserved green plant tissues pigment bleaching has also been commonly associated with freezing damage. This bleaching is probably free radical mediated (Benson, 1990). In addition, reduction in catalase activity, suggesting free radical activity has been observed, in a series of plants exposed to chilling (MacRae *et al.*, 1986). It is clear that freeze/chilling induced free radical activity/damage occurs in both plant and animal systems. It is therefore to be expected that a similar response could be anticipated in cryopreserved algae. The importance of photooxidative damage, catalase activity, chlorophyll fluorescence, electrolyte leakage and ethylene evolution may all be indicative of degrees of chilling tolerance.

1.11.5 Antioxidants

Antioxidant defence to oxidative stress is achieved by both enzymatic and non-enzymatic reactions. Vitamins C (ascorbic acid) and E (α-tocopherol) and vitamin precursors (*e.g.*, carotenoids) that reduce the rate of initiation or prevent the propagation of free radicals are notable non-enzymatic antioxidants (Kitts, 1997; Halliwell *et al.*, 1995). In plant chloroplasts, the first line of defence, against free radical production are the carotenoid pigments (Benson, 1990; Armstrong & Hearst, 1996; Smirnoff, 1993). Carotenoids confer protection in plants by allowing excited chlorophyll molecules to pass on their energy to the carotenoid pigments in preference to O_2. Carotenoids are also able to quench singlet oxygen ($^1O_2$) which provides a significant protective pathway in non-photosynthetic membrane systems (Benson, 1990; Hagen *et al.*, 1993c).

Of the vitamins, vitamin E is especially important in the prevention of lipid peroxidation, whereas vitamin C reacts effectively with superoxide and hydroxyl radicals (Kitts, 1997). Vitamin C also plays an important role in reducing semi-stable chromanoxyl radicals and regenerating vitamin E (Kitts, 1997). However, for some vitamins, the oxidative/antioxidative balance may favour prooxidant activity under specific conditions (Kitts, 1997). In many cases, this activity has been attributed to the interaction of vitamins with transition metals under certain conditions, which results in the production of a superoxide ion [$O_2^{\cdot-}$ (radical anion)], a hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) from the Fenton reaction (Kitts, 1997). The physiological significance of this potential prooxidant activity of nutrient vitamins remains to be determined (Kitts, 1997). Vitamin E is an effective free radical scavenger due in part to its lipophilic nature (reviewed by Benson, 1990). The phytol chain of vitamin E allows it to become embedded into the lipid layers of membranes, potentially at sites of free radical attack (reviewed by Benson, 1990). Vitamin E has also been found to quench singlet oxygen $^1O_2$ (Traber & Packer, 1995; Packer & Suzuki, 1993). Regeneration of vitamin E within the cell is believed to occur via the reduction of α-tocopherol quinone by ascorbic acid (vitamin C) back to α-tocopherol (Kitazawa *et al.*, 1997; Packer & Suzuki, 1993; Kunert & Ederer, 1985; Packer *et al.*, 1979).

Superoxide dismutase (SOD) is a general term for different families of metalloenzymes, which contain either manganese or iron, or copper and zinc as their prosthetic metals (Benson, 1990; Scott, 1997). In plant tissues most SOD is generally located in the chloroplasts, in non-photosynthetic tissue SOD is generally associated with cytoplasm and mitochondrial membranes (Halliwell, 1982). The antioxidant enzymes superoxide dismutase catalyse the conversion of $O_2^{\cdot-}$ to hydrogen peroxide (H_2O_2) whilst glutathione peroxidase (GSH-Px) metabolises the H_2O_2 to H_2O in the cell cytosol and endoplasmic reticulum. Antioxidant protection is also provided by catalase, a haeme containing enzyme which catalyses the breakdown of H_2O_2 and the oxidation of hydrogen donors (Packer, 1984). H_2O_2 and catalase are found in many types of cells and tissues in both plants and animals. In plants the production of H_2O_2 is by the SOD mechanism and in photorespiration (Benson, 1990; Scott, 1997). Several different peroxidases catalyse the dissipation of H_2O_2. Peroxidases also have several other functions in plant tissues including the degradation of indoleacetic acid (and are therefore important in the regulation of plant growth) and the promotion of lignification reactions (Halliwell, 1982; Omran, 1980).

Glutathione reductase is found in the soluble fraction of both prokaryotic and eukaryotic cells (Goldberg & Spooner, 1983). The primary role of the enzyme is apparently in the maintenance of the intracellular reduced glutathione (GSH) concentration which is required for the reduction of oxidised protein thiol groups (Goldberg & Spooner, 1983). The activity of the enzyme can be assayed spectrophotometrically (Goldberg & Spooner, 1983; Parihar *et al.*, 1997).

Glutathione is a low molecular weight tripeptide sulphur compound containing a thiol group (S-H) in the cysteine moiety (GSH) (Scott, 1997). The molecule functions to protect the cell by protecting oxygen sensitive membranes and other proteins from oxidative degradation (Halliwell, 1997; Halliwell, 1996a,b; Packer, 1984; Halliwell, 1991; Panigrahi *et al.*, 1996; Viguie *et al.*, 1993; Evans *et al.*, 1997). GSH confers protection by providing a preferential substrate for S-H oxidation with GSSG (oxidised glutathione) and water as the products of the reaction (Benson, 1990; Scott, 1997). However, excessive GSSG may be toxic to cells and its accumulation is controlled by

glutathione reductase which catalyses its degradation (reduction) and recycling into the protective GSH molecule (Benson, 1990; Scott, 1997). GSH may also protect the cell by removing H_2O_2 in conjunction with an ascorbate recycling reaction (Halliwell, 1982).

Oxidation of protein sulfhydryl (SH) groups has been proposed as a primary reaction leading to membrane damage in plant cells affected by ozone damage (Chevrier *et al.*, 1988). The action of ozone in the oxidation of total sulfhydryl groups has been demonstrated to be due to a large decrease in cellular protein SH content with a lesser reduction in non-protein SH (Chevrier *et al.*, 1988). It has been proposed that this is due to protein SH groups being more accessible to ozone than non-protein SH groups (Chevrier *et al.*, 1988). Since the first site encountered by the ozone is the plasma membrane it has been suggested that ozone primarily attacks the sulfhydryl groups of the membrane proteins, with the small amount of non-protein SH oxidised due to the penetration of ozone or reactive species into the cells (Chevrier *et al.*, 1988). Oxidation of the sulfhydryl groups is likely to lead to alterations of membrane properties, ionic imbalance and disturbance of metabolic processes which may impair subsequent growth (Chevrier *et al.*, 1988).

1.11.6 The importance of antioxidants in low temperature biology and storage

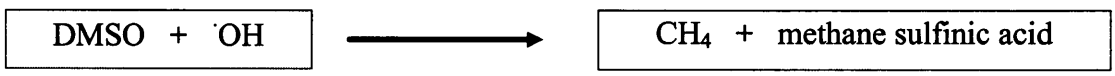
Organ transplantation frequently involves storage of the donated organ at low temperatures whilst it is transported to a suitable recipient. Oxygen derived free radicals have been implicated in cellular damage occurring immediately after periods of ischaemia at normal temperatures. Free radical scavengers, *e.g.*, DMSO, SOD, catalase and plant extracts with antioxidant capability, have been demonstrated to reduce the severity of events associated with free radical activity in a number of medical (Sarkar *et al.*, 1996; Smith *et al.*, 1996; Saugstad, 1996; Fuller & Green, 1986) and seed banking scenarios (reviewed by Benson, 1990).

1.11.7 Analytical techniques used to investigate free radical activity and cell damage

One of the consequences of lipid peroxidation is the production of breakdown products (Harding & Benson, 1995; Benson *et al.*, 1992a,b; Benson & Roubelakis-Angelakis, 1994). In senescent plants it has been reported that the decrease in chlorophyll levels and alteration in membrane permeability were accompanied by the accumulation of 2-thiobarbituric acid (TBA) reactive products, *e.g.*, malondialdehyde (MDA) (Merzlyak & Hendry, 1994). The basal level of lipid peroxidation in intact plant tissues is thought to reflect the activities of membrane electron transport chains, production of oxygen radicals by enzymes as well as the efficiency of systems which eliminate activated oxygen species and lipid peroxidation products (Merzlyak, 1989; 1990). Environmental factors including high irradiance, low and high temperature or desiccation promote free radical metabolism and induce lipid peroxidation in plants (Merzlyak, 1989; Merzlyak *et al.*, 1992). In addition to the volatile hydrocarbons produced as a consequence of lipid peroxidation malondialdehyde (MDA) is produced. MDA is a readily detectable product of lipid peroxidation and may be used as an indicator of freezing/chilling injury (Harding & Benson, 1995; Benson *et al.*, 1992a,b; Benson & Roubelakis-Angelakis, 1994; Benson & Withers, 1987). The ability of MDA to form a coloured complex with thiobarbitauric acid (TBA) allows the use of colorimetric and fluorimetric techniques for the detection of MDA by assaying for TBA complexes (Harding & Benson, 1995). However, it is worth noting that TBA will react with other lipid peroxidation products, hence the assay is more correctly expressed on the basis of general TBA activity (Harding & Benson, 1995). The production of volatile hydrocarbons is a further consequence of free radical activity, notably, lipid peroxidation.

It is possible to monitor hydrocarbon production by gas chromatography (Harding & Benson, 1995). This permits the non-invasive monitoring of the compounds evolved when plant and animal tissues are exposed to stress (Benson & Withers, 1987). Ethylene, a plant hormone, is widely used as an indicator of plant stress and studies have shown that ethylene evolution increases when plant tissues are subjected to a variety of stresses including wounding and pathogen attack (Benson & Withers, 1987). Ethylene biosynthesis is dependant upon membrane integrity and thus may be expected to cease

when stress is superseded by damage and subsequent cellular disruption (Benson & Withers, 1987). Under free radical mediated breakdown of damaged or senescent tissues radical reactions involved in lipid peroxidation can lead to the production of a number of volatile compounds (*e.g.*, ethane, propane, butane and pentane) (Benson & Withers, 1987). The reactive nature of free radicals results in the production of cascades of damaging chain reactions. The cryoprotectant DMSO acts as a hydroxyl radical (·OH) scavenger, releasing methane (CH_4). This permits the measurement of free radical activity through the monitoring of methane production from a sample in the presence of DMSO (Benson & Withers, 1987). Each of these volatile compounds may be collected when tissue is retained in sealed culture vials. The volatile component of the vials head space (CH_4) can then be analysed using gas chromatography (Benson & Withers, 1987; Harding & Benson, 1995) (see below for a summary).



1.11.8 Protective sulfhydryl groups

Sulfhydryl groups can be divided into two groups the non-protein thiols which are represented by very low molecular weight compounds, aminoacids (cysteine) or glutathione or by high molecular weight thiols, exclusively represented by protein thiols (protein bound sulfhydryls) (Faure & Lafond, 1995). Sulfhydryls in proteins play a number of important roles including: the conformation of structure, acting as the catalyst at the reactive site of numerous enzymes (*e.g.*, thiol protease), binding for the substrate of some enzymes, binding of some subunits, conformational change linked with allosteric processes and in a protective role against free radical damage (Faure & Lafond, 1995). Glutathione, a major non-protein sulfhydryl compound, has been linked with radioprotective action. Hydroxyl radicals (·OH) reacts with protein SH groups, particularly when they are generated near the SH sites. The more diffusible species, hydroperoxyl and superoxide radicals, can be rapidly scavenged by protein thiol groups, promoting numerous authors to hypothesise that protein thiol could be an intermediate reservoir which can protect against free radical attack (Faure & Lafond, 1995).

In medical investigations, ethanol induced gastric ulceration has been found to be associated with a significant reduction in non-protein sulfhydryl group levels in the stomach (Moghadasian & Godin, 1996). The reported ability of honey to protect against gastric haemorrhagic lesions is believed to be mediated through sulfhydryl sensitive processes (Ali & Al-Swayeh, 1997). Honey is thought to act by preventing depletion of endogenous non-protein SH and has, in addition, been reported to have antioxidative properties (Ali & Al-Swayeh, 1997). Furthermore, in mammalian studies of the effects of low temperature and freezing on tissues GSH and selenomethionine have been implicated as having a role in protecting tissues at freezing temperatures (Karl *et al.*, 1982; Matthes *et al.*, 1981; Armitage *et al.*, 1981). The efficiency of GSH mediated free radical scavenging has been thought to be improved by the presence of selenium (Richter & Armitage, 1985). Supplementation of tissues with fresh GSH has also been attributed to improved preservation of coronary endothelial function after prolonged cold storage (Kevelaitis *et al.*, 1997). Addition of fresh GSH to thawed bull spermatozoa has also been suggested to improve the quality of spermatozoa recovered after cryopreservation (Tegeler & Schuelke, 1992). GSH has been associated with the removal of hydrogen peroxide from the mitochondria (Ishikawa *et al.*, 1993a,b).

In addition, ice nucleators have been implicated as a component of the mechanism for freezing tolerance in winter rye (Brush *et al.*, 1994). Ice nucleators, have been demonstrated to be complexes of proteins, carbohydrates, and phospholipids in which both disulfide bonds and free sulfhydryl groups are important (Brush *et al.*, 1994).

1.11.9 Exogenous additives and applications in free radical research and damage avoidance

In addition to the cellular antioxidants, it is possible to affect free radical levels by removing catalysts involved in their synthesis, or the addition of exogenous antioxidants. Iron chelating compounds, *e.g.*, desferrioxamine, have been employed to suppress oxidative stress in mammalian transport organs subjected to cold storage (Benson *et al.*, 1995; Fuller & Green 1986; Gower *et al.*, 1989b,c). Desferrioxamine, a powerful Fe^{3+} chelator is able to prevent harmful transition metal chemistry, suppressing

oxidative stress encountered in tissues (Benson *et al.*, 1995) and it has been successfully used to reduce lipid peroxidation in cold-treated rabbit kidneys (Green *et al.*, 1986b; Gower *et al.*, 1987b).

Iron, a transition metal, plays a major role in the formation of oxygen radicals, the production of lipid peroxides and specific free radical degradation of proteins (Benson *et al.*, 1995). The transition metal cations (commonly ferrous or cuprous) produce active oxygen species via the Fenton reaction, most commonly through their action in conjunction with H_2O_2, producing the hydroxyl anion and the hydroxyl radical ($^{\cdot}OH$) via a one electron reduction (Benson *et al.*, 1995). The superoxide radical $O_2^{\cdot-}$ is also closely associated with the Haber-Weiss reaction, leading to reduction of Fe^{3+} salt and the further production of hydroxyl radicals ($^{\cdot}OH$) from H_2O_2 and Fe^{2+} (Pierre, 1995; Benson, 1990; Benson *et al.*, 1995) (see above). It can be seen that the damaging role of transition metals in lipid peroxidation is due to their ability to catalyse the formation of the highly toxic hydroxyl radical; the peroxides resulting from hydrogen abstraction by the lipid peroxide radical can also participate in iron mediated reactions which may ultimately lead to the production of the toxic aldehydes, malondialdehyde and hydroxyalkenals (Benson *et al.*, 1995).

Further evidence for the importance of Fe^{2+} has been attained from studies on *H. pluvialis* where acetate-induced astaxanthin biosynthesis can be activated by oxidative stress, promoted through the addition of Fe^{2+} and H_2O_2 (Kobayashi *et al.*, 1993a,b). The accompanied cyst/aplanospore formation and astaxanthin formation is enhanced by the addition of acetate and Fe^{2+} ions (Kobayashi *et al.*, 1993b). Encystment and astaxanthin formation has previously been implicated in the natural response of *H. pluvialis* to oxidative stress (Chaumont & Thepenier, 1995; Hagen *et al.*, 1993b,c; Lotan & Hirschberg, 1995). In *H. pluvialis*, Fe^{2+} enhanced carotenogenesis could be inhibited through the addition of potassium iodide, a scavenger for hydroxyl radical, suggesting that hydroxyl radical formed by an iron catalysed Fenton reaction may be required for enhanced carotenoid biosynthesis (Kobayashi *et al.*, 1993b). In addition, the active oxygen species, singlet oxygen, superoxide anion radical, hydrogen peroxide, and peroxy radical, were found to be capable of enhancing carotenoid formation (Kobayashi

et al., 1993b). Oxidative stress has therefore been implicated in the activation of carotenoid biosynthesis in *H. pluvialis* (Kobayashi *et al.*, 1993b).

Further examples of the application of exogenous antioxidants to reduce oxidative injury includes the treatment of corals with exogenous antioxidants to prevent "coral bleaching" (Lesser, 1997). The addition of antioxidants where oxidative stress has been implicated as a mechanism of "coral bleaching", prevents the loss of symbiotic algae (*i.e.*, bleaching) in corals and other invertebrates containing photoautotrophic symbionts (Lesser, 1997). Furthermore, in the culturing of microaerophilic bacteria, which are extremely sensitive to oxidative stress, studies have shown that constituents of the culture media are a potential source of free radicals, via the autooxidation and/or photoxidation of the nutrient medium (Hoffman *et al.*, 1983; Duwat *et al.*, 1995). To improve the culturability of microaerophiles, activated charcoal supplementation of the growth medium has been adopted (Hoffman *et al.*, 1983; Hoffman *et al.*, 1979). The activated charcoal detoxifies free fatty acids and active oxygen species and prevents free radical oxidation of cysteine (Benson, 1990). An additional method of reducing photooxidation is through the addition of casein and activated charcoal to nutrient agar (Waterworth, 1969; George & Sherrington, 1984).

1.12 The present state of algal cryopreservation

Cryopreservation has been successfully employed in a number of biological systems for the long-term storage of viable reproductive material (*e.g.*, germplasm in sperm/egg and embryo banks) (Ashwood-Smith & Farrant, 1980; Kartha, 1985b). The significant resources required for the *ex situ* maintenance of the entire holdings of major collections by serial subculture (Pringsheim, 1946) and the identification of routine serial subculture as being suboptimal in service culture collections has stimulated several of the largest protistan collections to develop methods suitable for the long-term preservation of their holdings (McGrath *et al.*, 1978; Morris, 1976a; Watanabe *et al.*, 1992; Bodas *et al.*, 1995). Although cryopreservation offers a long-term method for conserving algal cultures (Day *et al.*, 1997), their diverse morphological and physiological characteristics, have compounded the development of cryopreservation protocols.

To date only a limited range of algae have been successfully cryopreserved (Morris 1981, Morris 1978; Day, 1997) with many of the currently cryopreserved strains (350 of the 400 stored at the CCAP) belonging to two algal groups the Chlorococcales and the prokaryotic Cyanobacteria (Morris, 1978). When compared with seed banking methods of higher plants, low temperature storage in algal and protozoan collections is relatively restricted.

The majority of algal strains currently cryopreserved, have been preserved using two-step cooling methodologies adapted from higher plant and mammalian tissue preservation (see section 1.9.5). Algae have been deemed feasible to preserve by employing penetrative cryoprotectants, *e.g.*, DMSO or methanol (Fleck *et al.*, 1996). In addition, non-penetrating cryoprotectants including sucrose have been employed in protocols to freeze the conchocelis phase of *Porphyra linearis* (Arbault *et al.*, 1990). Some algal strains require the use of specific cryoprotectants to guarantee post-thaw viability, DMSO was determined to be the most suitable cryoprotectant for the prokaryote *Microcystis aeruginosa* (Box, 1988), however, for the marine Prasinophyte *Tetraselmis suecica*, glycerol was determined to be the least cytotoxic and most protective of the cryoprotectants tested (Fenwick & Day, 1992). Information on the toxicity of cryoprotectants employed is extremely important in obtaining viability after biological freezing of algae. In six taxonomically diverse marine microalgae evaluated for their tolerance to the cryoprotectants dimethyl sulfoxide (DMSO) and methanol all the species were sensitive to the concentration of cryoprotectant (Canavate & Lubian, 1994). In addition, different species were reported to respond differently when exposed to concentrations of DMSO and methanol (Canavate & Lubian, 1994).

In addition to cryoprotectant treatment optimisation of post-thaw viability of *Scenedesmus subspicatus*, was found to be specifically influenced by factors including pre-culture conditions and cooling rate (Benhra *et al.*, 1994). Optimisation of cryopreservation protocols may be also achieved though the development of specific pre- and post-culture regimes including culture under low light (17-22 μmol photon m^{-2} s^{-1}) (Beaty & Parker, 1992) or on agar media. Furthermore, culturing of algae at suboptimal temperatures has been linked to an increase in the likelyhood of their

surviving cryopreservation (Morris, 1976b) and cold acclimation has been demonstrated to permit higher levels of post-thaw viability in both freshwater (Morris, 1976b) and marine microalgae (Ben-Amotz & Gilboa, 1980). However, although it has been suggested by Morris (1981) that the improved post-thaw viability may be due to an increase in unsaturation in membrane phospholipid fatty acid composition, this has not been conclusively demonstrated for the algae, presenting the possibility of additional factors (*e.g.*, physiological or ultrastructural changes) promoted by cold acclimation enhancing cell survival post-thaw (Morris, 1981).

Algal response to two-step cryopreservation has also been found to be influenced by culture age and life history stage (Canavate & Lubian, 1997a; Morris, 1981). In five marine microalgae Canavate & Lubian (1997a) found that higher post-thaw viability levels were generally attained from older cultures, particularly when the cryoprotectant concentration and salinity conditions were suboptimal. Under optimal conditions this effect decreased or disappeared (Canavate & Lubian, 1997a). Older cultures, often lack vacuoles and with age their lipid content changes possibly favouring successful cryopreservation (Morris, 1981). Morris (1981) found that the lipid content and the presence/absence of a vacuole influenced post-thaw viability *Chlorella*. However, no advantage was reportedly gained, from collapsing the gas-vacuoles of *Microcystis aeruginosa* prior to freezing (Box, 1988). In studies on the cryotolerance of selected marine algae the general survival of algae on cooling rate appeared to decreased proportionally to the concentration of DMSO or methanol employed (Canavate & Lubian, 1995a). The nutritional mode of an alga prior to freezing was demonstrated to influence post-thaw viability levels of *Chlorella* (Morris *et al.*, 1977). However, the nutritional mode of the alga *Tetraselmis suecica* was reported to be of secondary importance to the cryoprotectant and cooling protocol employed, in attaining optimal survival (Fenwick & Day, 1992).

Further, to the effects of cryoprotectants and pre-growth conditions, cooling rate may also influence post-thaw viability. Cooling rate has been observed to affect post-thaw survival in *Chlorella*, where two strains of *Chlorella* have been found to yield different post-thaw viability levels over a range of cooling rates (Morris 1976a). In addition, the rate of cooling was observed to affect post-thaw viability in *Chlorella* 211/8h, where the

highest levels of viability were obtained at the faster cooling rates (Morris, 1976a). In addition, *Euglena gracilis* has been demonstrated to be extremely sensitive to cooling rate, with excessively fast or slow cooling rates reducing post-thaw viability (Fleck *et al.*, 1997b). In studies by Canavate & Lubian (1995b) on selected marine algae which employed ice seeding to avoid unpredictable supercooling, seeding was not reported to improve post-thaw viability.

To assess post-treatment viability in algae a variety of approaches have been employed including: vital staining (Arbault & Delanoue, 1994), changes in morphological appearance including de-pigmentation and/or a contraction of cells (Fleck *et al.*, 1997a; Arbault *et al.*, 1990), loss/retention of motility and biochemical methods (Arbault & Delanoue, 1994). A further approach to quantify the survival of algae cryopreserved on agar slants after freezing and thawing employed direct observation of algal cells chlorophyll pigmentation (Bodas *et al.* 1995). Lag phases may, however, occur in the alga's recovery (Arbault & Delanoue, 1994; McLellan, 1989; Fenwick & Day, 1992) and this may limit the effectiveness of many viability assessments which rely on rate of increase in cell numbers (Watanabe & Sawaguchi, 1995), these techniques should therefore only be termed indexes of viability. Absolute measurements of viability may only be determined by the measurement of cellular division and/or regrowth. The most commonly employed of theses techniques involve growth in or on solidified medium or the use of most probable number techniques (Day & McLellan, 1995b).

In addition, to the techniques employed to assess post-treatment viability in the algae, techniques have also been developed for the visualisation of lethal and non-lethal events during the cryopreservation protocol itself. Cryomicroscopy has been successfully employed to study changes in the morphology of algae during freezing and thawing (Morris *et al.*, 1986; Fleck *et al.*, 1997a; Roberts *et al.*, 1987; Roberts *et al.*, 1988). During slow freezing of *Cylindrocystis brebissonii* and species of *Micrasteria* extensive shrinkage of the protoplast was observed (Morris *et al.*, 1986). However, following freezing and thawing from -25°C non-viable cells of *C. brebissonii* remained osmotically responsive (Morris *et al.*, 1986). The application of faster cooling rates induced intracellular ice formation, however, the critical cooling rate varied with cell-type and was determined by cell volume and surface area (Morris *et al.*, 1986).

Cryomicroscopy also permits observation of the formation of intracellular gas bubbles during thawing (Morris *et al.*, 1986). The application of cryomicroscopy in conjunction with electron microscopy permits: observation of morphological responses of an alga to freezing, followed by rapid fixation for subsequent observation using electron microscopy to ascertain the sites of ultrastructural damage (Roberts *et al.*, 1987; 1988).

Although, many algae have been demonstrated to be freeze-recalcitrant when frozen in the absence of cryoprotective agent (Arbault & Delanoue, 1994), there are reports of algae which have been successfully cryopreserved by two-step cooling, in the absence of cryoprotectant (Canavate & Lubian, 1997b; Canavate & Lubian, 1995a; Day *et al.*, 1997). However, preservation of algae at low temperatures remains difficult and many algae remain freeze-recalcitrant although the ability of many algae to withstand cooling to high subzero temperatures both with and without cryoprotectant presents the possibility of successfully preserving these organisms by cryopreservation.

Cryopreservation protocols have largely followed conventional two-step methodologies with protocols being predominantly developed empirically (Table 1.5). Presently, many microalgae, (particularly those which have larger cell size and/or more complex morphology), are (or still remain) freeze-recalcitrant to conventional cryopreservation methodologies (Morris, 1981; Morris *et al.*, 1986). However there are increasing reports of successful cryopreservation of algae using both conventional two-step and more novel procedures (Table 1.5). In addition, to the reports of successful cryopreservation of previously freeze-recalcitrant algae, the alga *Undaria pinnatifida* has been found to have a limited duration of survival after storage at -80°C and at -196°C of only 9 and 4 days respectively (Arbault *et al.*, 1990).

A further, important consideration in the development of preservation procedures for use within culture collections, is that the method permits efficient handling and storage of a large numbers of algal strains (Bodas *et al.*, 1995). The UTEX Culture Collection has developed a procedure in which each culture is grown, prepared for freezing, cryopreserved, thawed and shipped on the same agar slant maintained in a 2 ml cryovial. These algae may also benefit from pre-culture on the agar slope and the reduced light levels within sealed cryovials prior to cryopreservation.

Table 1.5 Reports of successful cryopreservation of previously freeze-recalcitrant algae

Alga	Method	Reference
Dunaliella tertiolecta	two-step	Hirata *et al.*, 1996
Laminaria digitata	two-step	Vigneron *et al.*, 1997
Chlorella spp.	two-step	Canavate & Lubian, 1997a
Chaetoceros gracilis	two-step	Canavate & Lubian, 1995a
Tetraselmis chuii	two-step	Canavate & Lubian, 1995a
Nannochloris atomus	two-step	Canavate & Lubian, 1995a
Nannochloropsis gaditana	two-step	Canavate & Lubian, 1995a
Rhodomonas baltica	two-step	Canavate & Lubian, 1995b
Isochrysis galbana	two-step	Canavate & Lubian, 1995b
Cylindrocystis brebissonii	two-step	Morris *et al.*, 1986
Dunaliella tertiolecta	encapsulation/dehydration	Hirata *et al.*, 1996
Laminaria digitata	encapsulation/dehydration	Vigneron *et al.*, 1997

The investigations into the suitability of cryopreservation for the long-term maintenance of algal culture have indicated that where successful cryoprotocols have been developed it offers a robust and valuable technique (Day *et al*, 1997). It is therefore desirable to enhance current understanding of the mechanisms of cryoinjury and damage with the view to cryopreserving presently freeze-recalcitrant algae. However, although a number of authors have reported on the problems of the cryopreservation of lower eukaryotic and prokaryotic algae (McLellan *et al.*, 1991) including the effect of relationships between phylogenetic lines, habitats, cryoprotectants, an alga cryopreservability (Beaty & Parker, 1992). Considerable further work is required to gain a better understanding of mechanisms of cell damage and recovery in cryopreserved algae. The clear variations between the requirements of different algal strains, necessary, to achieve successful cryopreservation is likely to compound the successful preservation of these organisms and makes the development of a single cryopreservation protocol unlikely.

1.13 Project objectives

This project entitled "Mechanisms of Cell Damage and Recovery in Cryopreserved Freshwater Protists" aimed to elucidate mechanisms of lethal and sub-lethal cell damage in cryopreserved protists, particularly in the microalgae.

Specific objectives of investigation included:

- An investigation of the suitability of general cryobiological techniques, being applied to microalgae.
- The development of cryopreservation strategies for different microalgae phenotypes belonging to different taxonomic groupings and originating from different ecological niches.
- The development and application of novel investigative and biochemical techniques for the study of cryoinjury (including flow cytometry and microscopy).
- The application of novel cryopreservation techniques for the long-term conservation of microalgae.
- The investigation of free radical mediated events and their control at the cellular level and by exogenous routes in specific microalgae.

Chapter 2.

General materials and methods.

Contents **Page No.**

2. General materials and methods

2.1 Cultures

Eleven protistan and cyanobacterial strains were selected for study from the Culture Collection of Algae and Protozoa (CCAP) (Tompkins *et al.*, 1995). These are listed below in sections 2.1.1-2.1.2. The algal nomenclature employed follows the taxonomic classification of Bold & Wynne (1985).

2.1.1 Prokaryotes

2.1.1.1 Division Cyanophyta

Two freshwater members of the Cyanophyceae or "blue-green algae" were selected: *Microcystis aeruginosa* Kützing emend. Elenkin CCAP 1450/8 and *Anabaena cylindrica* Lemmermann CCAP 1403/2B (Table 2.1).

2.1.2 Eukaryotes

2.1.2.1 Division Chlorophyta

The freshwater eukaryotic unicellular alga *Haematococcus pluvialis* Flotow CCAP 34/8 was selected for study and maintained as distinct cultures, of either, motile or aplanospore stage cells. In addition, the brackish/marine alga *Enteromorpha intestinalis* (L.) Link CCAP 320/1 was also chosen (Table 2.1).

2.1.2.2 Division Euglenophyta

Freshwater members of the Euglenophyta: *Euglena gracilis* Klebs CCAP 1224/5Z, *Euglena gracilis* var. *saccharophila* Klebs CCAP 1224/7b, *Euglena mutabilis* Schmitz CCAP 1224/40 and the protozoon *Astasia longa* Pringsheim CCAP 1204/17A (Table 2.1) were selected.

2.1.2.3 Division Chrysophyta

2.1.2.3.1 Class Bacillariophyceae (diatoms)

Two freshwater diatoms: *Stephanodiscus hantzchii* Grunow in Cleve & Grunow CCAP 1079/4, and *Cyclotella pseudostelligera* Hustedt CCAP 1070/3 were selected (Table 2.1).

2.1.2.3.2 Class Xanthophyceae

The freshwater xanthophytic alga *Vaucheria sessilis* (Vaucher) De Candolle ex Collins CCAP 745/1C was selected (Table 2.1).

2.2 Routine culture and recovery media

Appropriate media for each alga were prepared in advance (Tables 2.2-2.7). Wherever possible, to ensure consistent quality and for ease of production, media were prepared from stock solutions. Stock solutions for BG11, DM, EG, JM and EG:JM were prepared in deionised water (dH_20), f/2 prepared in filtered natural sea water. Stock solutions were stored and refrigerated at 4°C, except JM stock solution 9 which was stored at room temperature.

Media were always prepared by the addition of stock solutions to a large proportion of the final volume of the medium, which was then made to volume with dH_20, except for f/2 which was prepared using filtered natural sea water. Media pH was adjusted using 1M HCl or 1M NaOH prior to being dispensed into suitable culture vessels (15 ml test-tubes, Schott bottles or 100 ml conical flasks) and then autoclaved at 121°C (101 kPa). for 15 min. Chemicals were obtained from BDH (BDH Chemicals, UK) or Sigma (Sigma Chemical Co., USA) unless otherwise stated and wherever possible AnalaR grade chemicals were selected. Solidified media were prepared by the addition of 1.5% (w/v) Bacteriological Agar (Oxoid L11) to the appropriate liquid media.

Table 2.1 Media used for culture maintenance

Organism	Strain No.	Culture Medium
Microcystis aeruginosa	CCAP 1450/8	BG11
Anabaena cylindrica	CCAP 1403/2B	JM
Haematococcus pluvialis	CCAP 34/8	EG:JM, JM
Enteromorpha intestinalis	CCAP 320/1	f/2
Euglena gracilis	CCAP 1224/5Z	EG:JM, JM
Euglena gracilis var. *saccharophila*	CCAP 1224/7B	EG, EG:JM
Euglena mutabilis	CCAP 1224/40	EG:JM
Astasia longa	CCAP 1204/17A	EG
Stephanodiscus hantzchii	CCAP 1079/4	DM
Cyclotella pseudostelligera	CCAP 1070/3	DM
Vaucheria sessilis	CCAP 745/1C	JM

Table 2.2 Blue-Green medium (BG11)

Stock No.	Compound	volume^{-1}
		litre (l)
1)	$NaNO_3$	15.0 g
		500 (ml)
2)	K_2HPO_4	2.0 g
3)	$MgSO_4.7H_2O$	3.75 g
4)	$CaCl_2.2H_2O$	1.80 g
5)	Citric acid	0.30 g
6)	Ammonium ferric citrate	0.30 g
7)	EDTA (Na_2 Salt)	0.05 g
8)	Na_2CO_3	1.00 g
9) Trace metal stock solution		litre (l)
	H_3BO_3	2.86 g
	$MnCl_2.4\ H_2O$	1.81 g
	$ZnSO_4.7\ H_2O$	0.22 g
	$Na_2MoO_4.2\ H_2O$	0.39 g
	$CuSO_4.5\ H_2O$	0.08 g
	$Co(NO_3)_2.6\ H_2O$	0.05 g

The medium was prepared using 100.0 ml stock solution (1), 10.0 ml stock solutions (2)-(8) and 1.0 ml stock (9) per litre. BG11 was adjusted to pH 7.1 prior to sterilisation (Stanier *et al.*, 1971; Tompkins *et al.*, 1995).

Table 2.3 Diatom medium (DM)

Stock No.	Compound	200 ml $^{-1}$
1)	$Ca(NO_3)_2.4H_2O$	4.0 g
2)	K_2HPO_4	2.48 g
3)	$MgSO_4.7H_2O$	5.0 g
4)	$NaHCO_3$	3.18 g
5)	EDTA (FeNa Salt)	0.45 g
	EDTA (Na_2 Salt)	0.45 g
6)	H_3BO_3	0.496 g
	$MnCl_2.4H_2O$	0.278 g
	$(Na_4)_6Mo_7O_{24}.4H_2O$	0.20 g
7)	Cyanocobalamin	0.008 g
	Thiamine HCl	0.008 g
	Biotin	0.008 g
8)	$NaSiO_3.9\ H_2O$ (Sigma S4392)	11.4 g

DM medium was prepared using 1.0 ml of each stock solution (litre^{-1}). DM was adjusted to pH 6.9 prior to sterilisation (Tompkins *et al.*, 1995; Beakes *et al.*, 1988).

Table 2.4 Euglena gracilis medium (EG)

Stock No.	Compound	litre $(l)^{-1}$
1)	$CaCl_2$	1.0 g

EG was prepared by the addition of Sodium acetate trihydrate (1.0 g), "Lab-Lemco" powder (Oxoid L29) (1.0 g), Tryptone (Oxoid L42) (2.0 g), Yeast extract (Oxoid L21) (2.0 g) and $CaCl_2$ stock soln. (10.0 ml) to deionised water ($litre^{-1}$) (Tompkins *et al.*, 1995).

Table 2.5 EG:JM medium

This medium was prepared using a 1:1 mixture of EG and JM. Media were mixed and then autoclaved (Tompkins *et al.*, 1995).

Table 2.6 f/2 medium (f/2)

Stock No.	Compounds	litre^{-1}
1) Trace metals stock solution (chelated)		
	EDTA (Na_2 Salt)	4.160 g
	$FeCl_3.6H_2O$	3.150 g
	$CuSO_4.5H_2O$	0.010 g
	$ZnSO_4.7H_2O$	0.022 g
	$CoCl_2.6H_2O$	0.010 g
	$MnCl_2.4H_2O$	0.180 g
	$Na_2MoO_4.2H_2O$	0.006 g
2) Vitamin mix stock solution		
	Cyanocobalamin	0.0005 g
	Thiamine HCl	0.1 g
	Biotin	0.0005 g

f/2 was prepared by the addition of KNO_3 (0.075 g), $NaH_2PO_4.2H_2O$ (0.00565 g) and 1.00 ml of each stock solution with filtered natural sea water to 1 litre (final volume) and adjusted to pH 8.0 (Tompkins *et al.*, 1995).

Table 2.7 Jaworski's medium (JM)

Stock No.	Compounds	per 200 ml
1)	$Ca(NO_3)_2.4\ H_2O$	4.0 g
2)	K_2HPO_4	2.48 g
3)	$MgSO_4.7H_2O$	10.0 g
4)	$NaHCO_3$	3.18 g
5)	EDTA (FeNa Salt)	0.45 g
	EDTA (Na_2 Salt)	0.45 g
6)	H_3BO_3	0.496 g
	$MnCl_2.4H_2O$	0.278 g
	$(Na_4)_6Mo_7O_{24}.4H_2O$	0.20 g
7)	Cyanocobalamin	0.008 g
	Thiamine HCl	0.008 g
	Biotin	0.008 g
8)	$NaNO_3$	16.0 g
9)*	$Na_2HPO_4.12\ H_2O$	7.2 g

* stored at room temperature.

JM was prepared by the addition of 1.0 ml of each stock solution per litre of deionised water (Tompkins *et al.*, 1995).

2.3 Conditions for culture maintenance and post-treatment recovery

2.3.1 Culture conditions

Algae were maintained at 15°C under a 12:12 h. light:dark regime. Illumination was provided by cool white fluorescent lamps with a photon flux density of 50 μmol m^2 s^{-1} at the surface of the culture vessel. During post-treatment recovery, cultures were maintained under standard condition with incubation temperatures of either 22°C or 15°C.

2.3.2 Maintenance regime

Sub-culturing of organisms was performed using aseptic microbiological techniques. For unicellular cell suspensions, an inoculum of 1 ml (2% v/v) was transferred from a late log/stationary phase culture into the appropriate fresh, sterilised medium. Transfer intervals varied between 2 weeks and 2 months depending on the growth rate of the organism. Cultures were maintained in 100 ml conical flasks containing 50 ml of the appropriate medium. Flasks were capped with foam bungs.

Filamentous organisms were transferred by teasing a 1-10% inoculum from the late log/stationary phase culture and transferring the inoculum with a sterile loop (Elkay, 10μl loop/needle) to fresh media. *V. sessilis* was maintained in 15 ml test tubes containing 10 ml media. Test tubes were capped with siliconised rubber bungs or plastic caps. *E. intestinalis* was maintained in 100 ml conical flasks capped with foam bungs containing 50 ml media. All cultures were maintained under standard culture conditions (see 2.3.1).

2.4 Cryostorage conditions

Long-term cryostorage of algal cultures was undertaken in the CCAP cryostore. Each strain was deposited in a 1.8 ml cryovial (NUNC) containing 0.5 ml of cell suspension. Vials were allocated a storage site in an aluminium inventory system (MVE, France). These were retained in wide necked bulk storage dewars (cryostats)(MVE XLC 230, or

equivalent). Nitrogen levels were monitored twice weekly and liquid nitrogen added manually as required. Storage was primarily, in liquid-phase nitrogen at -196°C, with direct contact between the inventory and liquid nitrogen at all times during normal storage (Day *et al.*, 1997).

2.5 Viability assessment

The following viability techniques include both cell regrowth measurements and assessments of viability. Vital staining, morphological appearance and motility were all used as indices of algal viability (McLellan, 1989; Fenwick & Day, 1992). However, absolute measurements of viability could only be determined using techniques involving cellular division and/or regrowth.

2.5.1 Dilution series for viability assessments

Treated cells were agitated with a sterile plastic pastette (1 ml) to separate aggregates of cells (*H. pluvialis*) and aseptically transferred to appropriate fresh, sterile, medium (final volume of 10 ml). Microscopical conformation of the separation of cell aggregates was performed. Aliquots (1 ml) of material were transferred to a further 9 ml of the appropriate media and this was repeated until a 1 in 1000 dilution had been achieved.

2.5.2 Vital/mortal stains and fixation

2.5.2.1 Fluorescein diacetate stain

Fluorescein diacetate (FDA) (Sigma, F 7378) was used as a viability stain in microscopical and flow cytometer viability assessments. Cells with functioning esterase activity cleave the stain which then fluoresces intensely yellow/green under UV illumination; non-viable cells appear colourless or red due to the autofluorescence of chlorophyll.

A stock solution of FDA, 0.1% (w/v) in 100% acetone or methanol was prepared for microscopical investigations. The stock solution was filtered (0.2μm filter, Sartorius,

Germany) and stored in a refrigerator at 4°C. The stain was diluted to 1µl per ml with the appropriate culture medium immediately prior to use. A few drops of the diluted stain were added to cells on a clean microscope slide and observed under UV illumination as described previously by Harding & Benson (1995).

For flow cytometry studies fresh FDA [0.1% (w/v)] was prepared by first dissolving FDA crystals in a few drops of acetone and making up to the final volume with methanol and filtered (0.2µm filter, Sartorius, Germany). The stain was added to the sample at 1µl per ml and incubated for 1min prior to being loaded into the flow cytometer.

2.5.2.2 Evans blue and neutral red stain

Evans blue (Sigma, E 2129) was used as a mortal stain. It only stains dead protoplasts, and is excluded from live cells by the presence of an intact cell membrane (Saga *et al.*, 1987). Neutral red (Sigma, N 7005), was used as a vital stain and stains live cells through the uptake and concentration of neutral red in their vacuoles (Saga *et al.*, 1987).

The staining dyes, Evans blue and neutral red, were prepared at 0.01% (w/v) in filtered (0.2µm filter) dH_20. A few drops of stain were added to the cells which were mounted on a microscope slide and a coverslip was lowered onto the specimen. Cells were then observed with a compound microscope (Saga *et al.*, 1987).

2.5.2.3 DAPI stain

Fixed cell samples were stained with DAPI [4,6-diamidino-2-phenylindole (Sigma, D 9542)], a fluorescent groove-binding probe for DNA. DAPI stock solution was prepared in advance by dissolving DAPI (1 mg) in 10 ml dH_20. The solution was filtered (0.2 µm filter, Sartorius, Germany) and stored refrigerated at 4°C (Porter & Feig, 1980). DAPI stock solution was then added to the cells (0.4 ml DAPI stock solution added to 3.6 ml fixed sample). Stained cell suspensions were filtered through a 0.2 µm Black Nuclepore placed on a 0.45 µm clear Nuclepore backing filter, pre-wetted with dH_20. The filter

was then blotted and mounted on a microscope slide smeared with immersion oil. A drop of oil was then added and a coverslip gently lowered over the sample which was then observed under UV illumination.

2.5.2.4 Fixatives

Cells were fixed with either 2.5% (v/v) aqueous formaldehyde solution, FAA (100 ml commercial formalin, 50 ml glacial acetic acid, 500 ml 95 % ethanol, 350 ml filtered dH_20) (Entwisle, 1987) or in buffered (0.1M sodium cacodylate buffer) glutaraldehyde [4% (w/v)].

2.5.3 Haemocytometer counts

Cell counts were performed on an improved Neubauer haemocytometer. Cell suspensions were transferred to both chambers of the haemocytometer using a Pasteur pipette by carefully touching the edge of the cover-slip with the pipette tip, allowing each chamber to fill by capillary action. Care was taken to ensure that chambers were neither under- nor over-filled. For each haemocytometer chamber, all cells within the 1 mm^2 centre square and 4 corner squares were counted.

Each square of the haemocytometer with the cover-slip in place represents a total volume of 0.1 mm^3 or 10^{-4} cm^3.

Cells per ml = the average count per $1mm^2$ × dilution factor × 10^4

Total number of cells = cells per ml × the original volume of cell suspension from which the sample was removed.

2.5.4 Plate counts

Aliquots (1 ml) of diluted cell suspensions were placed in sterile 50 ml Petri dishes (Sterilin), and the appropriate sterile medium containing 1.5% (w/v) agar (3 ml) was added at 35 °C. Petri dishes were gently agitated to mix the agar and cell suspension, distributing the cells within the agar. Once the agar had solidified the lids were placed

on the Petri dishes and sealed with Parafilm (American National Can, USA). They were then transferred to a recovery incubator and stored inverted until distinct colonies were noted. Colonies were counted on examination under a dissecting microscope (×25). In the case of organisms which were non-motile within the agar, one colony was assumed to arise from one viable cell (Fenwick & Day, 1992).

2.5.5 Increase in cell numbers

Diluted cells were recovered in a fixed volume of the appropriate fresh media and incubated for 7 days under standard conditions. The initial cell number and the cell number after 7 days were counted using a haemocytometer. The percentage viability (V), assuming that there was no lag phase, was calculated as follows (Watanabe & Sawaguchi, 1995):

$$V = (b - \alpha) / (a - \alpha) \times 100$$

"a" = the 7 day rate of increasing cell number pre-treatment
"b" = the 7 day rate of increasing cell number post-treatment
"α" = the remaining rate of non-viable cells, which remained intact and were therefore counted.

This allowed % viability (V) to be determined:

$$V = (b - 1) / (a - 1) \times 100$$

2.5.6 Chlorophyll *a* measurement

Measurements of chlorophyll levels were used as an index of viability after a period for bleaching had been allowed (>72 h.). Bleaching was due to photo-oxidation of chlorophyll and other photosynthetic pigments in non-viable cells.

2.5.6.1 Liquid culture unicellular suspension

Aliquots of cell suspension were centrifuged (1 min) to pelletise the cells at the base of a centrifuge tube. The supernatant was removed and the cells were re-suspended in solvent (methanol [MeOH] or 80% (v/v) acetone). Chlorophyll was cold extracted in the dark overnight or at 60°C for 20 min. The cells suspension was then centrifuged and the absorbance of the supernatant at 663 nm measured against a blank (MeOH or 80% (v/v) acetone) and the concentration of Chl. *a* determined as described by MacKinney, (1941).

2.5.6.2 Immobilised cells or filamentous material

A known wet weight of sample (filaments or 4 beads) were hand homogenised in extraction solvent (MeOH). The material was then boiled in a sealed tube, in the dark, at 80°C for 20 min. After centrifugation the absorbance of the supernatant was measured against a MeOH blank at 663 nm as above.

All measurements were recorded in triplicate.

2.5.7 Oxygen measurement using an oxygen electrode

The electrode (Rank, UK) was cleaned prior to the start of each experiment with tissue paper wetted with cleaning solution (n 3% NH_4OH) then rinsed with dH_20 and prepared for use.

The oxygen electrode was calibrated with oxygenated dH_2O, prepared by bubbling air through dH_2O for 30 min. Air-saturated dH_2O (= 0.75 μM oxygen in 3 ml dH_2O) was added to the electrode chamber with the stirrer running and the electrode chamber sealed. After a short period for equilibration, the air saturated base line was recorded on a chart recorder (set at $1cm.min^{-1}$), a few crystals of sodium dithionate ($Na_2S_2O_4$) were added and the zero oxygen base line recorded. The liquid was then removed from the electrode and the electrode and chamber were thoroughly washed with dH_2O.

Respiration and oxygen evolution rates were measured according to Whitelam & Codd (1983). Samples (3 ml) were placed in the electrode chamber, conditions were carbon non-limiting at pH 7 (0.1 ml $NaHCO_3$ (4 mM) per 3 ml media). Light illumination was constant and non-limiting for oxygen evolution steps (oxygen evolution due to photosynthesis) (440 μmol m^2 s^{-1}) and respiration rates were determined in the dark.

All experiments were performed in triplicate.

2.5.8 Flow cytometry

Treated and untreated cells were analysed in a FACStar Plus flow cytometer (Becton Dickinson, UK) set to trigger on forward scatter. Three parameters were measured: forward light scatter (FSC), 90° side-scatter (SSC) and fluorescence at 530 ± 30 nm. The laser power at 488 nm was set to 20 mW and all data were recorded on a log scale. The photomultiplier voltages were set at 400 V for SSC and 450 V for the fluorescence detector. The cytometer was aligned with 0.5 μm diameter Fluoresbrite YG fluorescent latex beads (Polysciences, USA). The nozzle diameter was 70 μm. For each sample, 5000 events were recorded. The FACStar Plus computing package was used to determine the proportion of cells that were fluorescently labelled with FDA and to display the data as single-dot plots or histograms. For each data set, unstained sub-samples were removed and used as negative controls to determine the baseline fluorescence of non-labelled cells (Pickup *et al.*, 1996). Cut off points were then determined as being areas where no background fluorescence was detected. In subsequent stained samples, it was possible to measure the proportion of population labelled with the viability stain.

2.5.9 Viability procedures for *V. sessilis*

Algal filaments were inspected for cytoplasmic streaming and wound healing pre- and post treatment using a Leitz Dialux 22 compound microscope. Normal filaments of this organism show characteristic wound healing after mechanical injury involving the cutting/breaking of the algal filament (Tornbom & Oliveira, 1993a,b). Wound healing is

a multistage process: Stage 1, a globular vesicle emerges from the wound site; this occurs approximately 1 min after wounding. Stage 2 involves the sealing of the wound, accompanied by an accumulation of large numbers of organelles at the wound site, this is normally observed between 4 and 8 min. post-wounding. Assuming successful completion of the first two stages, full recovery of normal cell wall morphology occurs (Tornbom & Oliveira, 1993a,b). Loss of cytoplasmic streaming and the capacity to recover from the cutting of the algal filament were considered to indicate that the alga had suffered significant damage. Thus, the wound healing response could be used as a convenient indicator of injury and recovery.

In addition to the above, treated filaments were transferred to fresh liquid or solid medium and incubated under standard conditions for 14 days. The material was inspected at 24 h. intervals. Filaments that bleached after the first 72 h., due to photo-oxidation of chlorophyll and other photosynthetic pigments, were considered to be dead.

2.6 Microscopy

2.6.1 Light microscopy

Live and fixed cells/filaments were examined using an Olympus BH-50 microscope equipped for bright field, phase-contrast, fluorescence and Normarski Differential Interference Contrast (DIC) microscopy. Cells/filaments were also observed using a Leitz Dialux 22 which was equipped for bright field, fluorescence microscopy and with filters which allowed the specimens to be viewed with polarised light. Cells were mounted on microscope slides over which a coverslip was gently lowered. Still photomicrographs were taken using an Olympus OM-2n, 35 mm camera

2.6.2 Cryomicroscopy

Cells in medium were placed in shallow wells (100-150 μm deep) or in drops of medium (100 -200 μl) directly on glass microscope coverslips (Chance Propper Ltd., No. 0). The top edge of the well or the outside edge of the cover slip was then lightly

coated with silicone grease M494 (Ambersil Ltd.) and the cells/filament were then sealed between two coverslips by placing a further glass microscope coverslip on top. This arrangement allowed a thin, unicellular layer of material to be examined without evaporation of the medium.

Images were collected using both video and still camera. Video images were collected with a colour video camera (JVC TK-1280E) onto a VHS video recorder (Ferguson). Still images were obtained using an Olympus OM-2n, 35 mm camera.

2.6.2.1 Planer CM3

The Planer CM3 Cryostage was attached to an Olympus BH-22 compound microscope equipped with bright field and phase-contrast. The stage was cooled by LN vapour with vapour delivery driven by head pressure in the supply Dewar via a heat sink (a copper coil immersed in LN). The sample was mounted and observed according to Fleck *et al.* (1997a).

2.6.2.2 Linkam BCS 196

The Linkam BCS 196 Cryostage (Linkam Scientific Ltd., UK) was mounted on either an Olympus BX-50 microscope or a Leitz Dialux 22 microscope with an adapter unit modified from a standard Leitz stage. The Olympus BX-50 was equipped for bright field, phase-contrast, fluorescence and Normarski, Differential Interference Contrast (DIC) microscopy.

The BCS 196 cryostage was mounted and centred in the field of view of the microscope before being secured. The stage was cooled by drawing LN from a 2 l Dewar (Linkam D2L) through a small bore tube, via a filter, by a cooling pump (Linkam LNP).

Samples were mounted and observed according Fleck *et al.* (1997a). The BCS 196 cryostage was used throughout with the ice "seeding" device disabled. Measurements of changes in cell/filament width and area were performed using a Linkam video text overlay unit VTO 232 according to Fleck *et al.* (1997a).

2.6.3 Electron microscopy

Algal samples were fixed at room temperature for experimentally determined time periods in a sequence of standard fixatives specific to each alga. Fixatives included: buffered paraformaldehyde [2% (w/v)] (0.1M sodium cacodylate buffer), buffered glutaraldehyde [2% (w/v)], buffered osmium tetroxide [2% (w/v)] and aqueous uranyl acetate [2% (w/v)]. Fixed samples were dehydrated in a graded ethanol series from 25 to 100% ethanol and in 100% ethanol.

2.6.3.1 Scanning electron microscopy

Fixed and fully dehydrated material was either critical point dried from liquid CO_2 (Polaron, UK) or freeze dried (Edwards, UK), coated with gold (Polaron, UK) and examined with a Jeol 100CX TEMSCAN (scanning mode) or a Jeol JSM-25S scanning microscope.

2.6.3.2 Transmission electron microscopy

Fixed and fully dehydrated material was embedded in Spurr resin (Spurr, 1969) for ultramicrotomy. Embedding was carried out in a graded resin series and in 100% Spurr. Samples were incubated at room temperature, overnight for each step, and finally cured at 60°C. Sections of the embedded material were cut on a Reichert OmU2 ultramicrotome and triple stained after conditioning in 0.5% (v/v) hydrochloric acid using a Reynolds' lead citrate [2% (w/v)] (Reynolds, 1963)/uranyl acetate [2% (w/v)]/Reynolds' lead citrate [2% (w/v)] protocol (Clarke *et al.*, 1993) and material was examined using a Jeol 100CX TEMSCAN (transmission mode).

Chapter 3.

Conventional cryopreservation approaches.

Contents **Page No.**

3.1 Introduction

Cryopreservation has frequently been adopted as the preservation method of choice in microbial culture collections (Kirsop & Doyle, 1991) and has been employed by most of the major algal culture collections (Morris, 1978; Watanabe *et al.*, 1992; Bodas *et al.*, 1995) (1.6). As discussed in 1.9.3, in viable material, stored at -196°C, normal cellular chemical reactions cease (Grout *et al.*, 1990), and this has permitted the cryopreservation of algal cell lines with no significant reductions in viability for up to 22 years of storage (Day *et al.*, 1997). Presently, however, only a relatively limited range of eukaryotic algae have been cryopreserved, with many of the successfully preserved eukaryotic algae belonging to the Chlorococcales (Day *et al.*, 1997) (1.12).

Where successful protocols are currently employed, they have largely been developed empirically and successful protocols have proven effective on the basis that they reduce osmotic stress, cold shock and potential damage by intracellular and extracellular ice formation, before and during freezing, and on thawing. However, as discussed in 1.9-1.11.9 there are a number of factors which contribute to the difficulty in developing successful cryopreservation protocols. In addition, algae are also subjected to a radically altered physical and chemical environment whilst in their frozen aqueous media, subjecting the cells, to a series of further stresses. These stresses, imposed on the cells during both freezing and thawing, may have wide ranging effects on cell physiology, morphology and ultimately viability (Grout & Morris, 1987; Morris, 1981).

In this chapter, the suitability of two-step cryopreservation techniques, using either uncontrolled or controllable cooling rate methodologies, have been investigated for the preservation of microalgae. Members of the algal classes Bacillariophyceae, Chlorophyceae, Cyanophyceae, Euglenophyceae and Xanthophyceae have been examined with the objective that an improved understanding of the effects, of two-step controlled rate cryopreservation on these model systems, will enable the development of improved cryopreservation methods for currently freeze-recalcitrant algae.

3.1.1 Development of robust cryopreservation protocols

Several methods have already been developed to cryopreserve a range of microalgae, however, no single protocol has been found to be universally satisfactory. Some strains are apparently unable to withstand cryopreservation using known methods, whilst others may be frozen successfully in the absence of cryoprotectant by plunging directly into liquid nitrogen (Day *et al.*, 1997). A two-step protocol (Cooling to an intermediate subzero temperature prior to plunging into liquid nitrogen) has been used to cryopreserve the majority of algal strains. Where this has proven successful, post-thaw viability levels of over 95% have been attained (Day *et al.*, 1997; Morris, 1978).

The preservation of algae with high survival levels is of key importance in the development of robust cryopreservation protocols. High post-thaw viability levels minimise the possibility of selecting a preservation-tolerant sub-population and reduces the incubation time required for re-growth of a preserved culture to a suitable density for distribution. Additional advantages include the prevention of genetic change in the preserved culture and reduction in labour costs compared with traditional, serial subculture. In addition, a robust protocol must be capable of being employed over a number of years with no variation in post-thaw viability levels and must satisfy a number of further criteria including: being readily reproducible, easily implemented, cost effective and be technically simple to facilitate technology transfer between personnel.

3.1.2 Investigation of the long-term storage of algae

Comparatively few long-term data-sets have been published in the scientific literature outlining the effects of prolonged cryostorage on microorganisms. Where storage of material is at high subzero temperatures (> -70°C), viability levels fall significantly over time (John & John, 1996). For organisms maintained in liquid or vapour-phase liquid nitrogen, viability levels should effectively be independent of storage duration measured in decades (Grout, 1995). In the present study *Haematococcus pluvialis* was examined for changes in post-thaw viability levels over a 12 month period of cryostorage.

3.1.2.1 The stability of a cryostat

Long-term preservation of cells in culture collections requires viable material to be maintained in a stable temperature regime for extended periods. It is possible, that if wide variations in temperature are encountered during cryostorage, cell damage may occur. Temperature variations may potentially arise during retrieval and deposit manipulations of stored cryovials, or, during the routine maintenance of a working cryostore.

Damage due to temperature fluctuations could theoretically occur in stored material if temperatures are raised above the limit of ice crystal growth -139°C (Morris, 1981). As discussed in 1.9.2 damage due to recrystallisation or devitrification could occur if temperatures were raised to a point at which small cubic ice (I_c) particles were able to minimise their surface to volume ratios by fusing or growing into larger, more stable hexagonal ice forms (I_h). Although this generally occurs at high subzero temperatures, recrystallisation has been detected as low as -130°C (Taylor, 1987).

3.1.3 Objectives

The investigation endeavoured to explore the effectiveness of routinely applying two-step controlled rate cooling methodologies to the algae, in addition, the practicality of long-term maintenance of the diverse range of microalgae held cryopreserved at the CCAP will be assessed.

3.2 Materials and methods

3.2.1 Organisms and culture regimes

Cultures selected for study are detailed in 2.1. Organism culture regimes and recovery conditions were performed as described in 2.2-2.3.

Filaments of the xanthophytic alga *Vaucheria sessilis* were sectioned into 12 mm lengths, then incubated under standard conditions for 48 h., prior to cryopreservation

(Fleck *et al.*, 1997a). *V. sessilis* sections were also pre-cultured in media supplemented with 0.09 M Sucrose or 1.2 M Sorbitol (Sigma, USA).

3.2.2 Cryopreservation procedures

Cryoprotectant chemicals employed were: dimethylsulphoxide (DMSO), methanol, ethylene glycol (EG), propylene glycol (PG) and glycerol (Sigma, USA). Final concentrations of 5 or 10% (v/v) cryoprotectant were used throughout with 5 or 15min. exposure at room temperature (RT/20°C), or at 0°C prior to cooling to subzero temperatures. The cryoprotectant was always added to the cell suspensions, in order to avoid excessive toxic shock during the mixing of the cryoprotectant solution and the cell suspension. Vials from liquid nitrogen (LN) were maintained for at least 1 h. in LN prior to thawing.

Vials containing 0.5ml of algae/cryoprotectant were frozen by either two-step uncontrolled rate or controlled cooling rate methodologies (2.4).

3.2.2.1 Two-step uncontrolled rate cooling

Vials containing cells for two-step direct cooling were first immersed in a Fryka Kältetechnic KB650 KR, industrial methylated spirits (IMS), bath (Camlab, UK) pre-cooled to -35°C. Cells were held at -35°C for 15 and 30 min. periods, prior to being plunged directly into LN. All material was thawed using a single-step thawing protocol involving the direct immersion of vials in a waterbath pre-heated to 40°C and agitated in the waterbath until the last ice crystals had melted (Day *et al.*, 1997). The thawed material was then aseptically transferred to appropriate fresh, sterile, medium (2.2), and used in the viability assays as outlined in 2.5. All treatments were performed in triplicate and errors are expressed as standard errors of mean.

3.2.2.2 Two-step controlled rate cooling

Vials containing cells for controlled cooling were cooled to their intermediate holding temperature using a Planer Kryo 10 programmable freezer (Planer, UK), two

programmable freezers were used: a Mk I large capacity unit and a Mk III low capacity embryo freezer. Cooling rates of -1°C $min.^{-1}$, -0.5°C $min.^{-1}$, and -0.3°C $min.^{-1}$ were employed. Vials were cooled to -35, -45 or -60°C and held at the pre-determined intermediate temperature for 15 and 30 min. periods, prior to being plunged directly into LN.

Vials were thawed following a one, or a two-step protocol. One-step protocols employed a single direct immersion in a pre-heated waterbath at 40°C. In two-step protocols vials were first allowed to warm slowly by holding in the air for 1min. vials were then rapidly warmed in a pre-heated 40°C waterbath.

All vials were agitated in the waterbath until the last ice crystals had melted (Day *et al.*, 1997). The thawed material was then aseptically transferred to appropriate fresh, sterile, medium (2.2), and used in the viability assays as outlined in 2.5. All treatments were performed in triplicate and errors are expressed as standard errors of mean.

3.2.3 Recovery from the cryostore

Vials which were maintained frozen for long-term investigations were transferred from the cryostore to a small Dewar containing liquid nitrogen (2.4) and transferred to the laboratory for thawing (3.2).

3.2.4 Measurement of temperature changes in CCAP cryostore

In the CCAP cryostore, the storage regime has been maintained by the twice weekly addition of liquid nitrogen for over 20 years (Day *et al.*, 1997). The effect of this maintenance regime on vials stored at the top, middle and bottom of the inventory system was monitored over a three week period. Thermocouples (RS Components) were inserted into 1.8 ml cryovials (NUNC, Denmark), containing 0.5 ml of water, via punctured cryovial caps. These vials were then placed in the centre of three cryo-storage drawers in the top, middle and bottom of the inventory system. This was then lowered into the cryostore. Liquid nitrogen was added to "top up" the cryostat, and the cryovial temperature allowed to equilibrate at -196°C before commencing logging of the internal

cryovial temperature. The temperature of each vial was logged at 10 min. intervals on a data-logger with data-logging software (DeCipher Plus v1.1) running on a lap-top computer (Opus Technology, UK) (Day *et al.*, 1997).

In addition, the effects of sample manipulation on stored vial internal temperatures, were monitored for vials positioned at the top, middle and bottom of the inventory system. Vials were prepared as above, after initial equilibration of the internal temperature, the temperature was recorded at 0.25 s. intervals during a series of simulated addition and retrieval procedures of vials at each storage location (Day *et al.*, 1997).

3.2.5 Exotherm measurement in cryovials

Exotherm data was obtained using a Planer Kryo 10 Mk III programmable freezer (Planer, UK). Thermal histories for the cryoprotocol were printed to show the programmed freezing regime, the thermal regime attained during the protocol in the freezing chamber and within the sample vial. Supercooling was confirmed in both Planer Kryo 10 programmable freezers by the random removal of vials at subzero temperatures during simulated freezing protocols.

3.3 Results

3.3.1 Two-step cooling using an IMS immersion bath

Two-step uncontrolled rate cryopreservation protocols were employed to freeze five different algal strains with varying degrees of success (Table 3.1). From these initial investigations it was demonstrated that *Haematococcus pluvialis* could be recovered from subzero temperatures with high levels of post-thaw viability (> 80%) (3.3.1.2).

Viability levels for *Microcystis aeruginosa* after 15 min. at -35°C reduced to 62% ± 2% of the untreated control, extension of the holding period by a further 15 min. further reduced viability to 50% ± 1.5%. The cryoprotectant exposure step (5% DMSO (v/v),

15min. exposure at 20°C) had no obvious effect on the viability of *M. aeruginosa*. Viability levels were 25% ± 1% after thawing from LN.

Table 3.1 Effects of uncontrolled two-step cooling on the post-thaw survival of selected strains

Organism	-35°C [a]	-196°C [b]	-196°C direct [c]
[d] *E. gracilis*	-	-	-
[e] *H. pluvialis*	+	+	-
[f] *V. sessilis*	-	-	-
[d] *M. aeruginosa*	+	+	-
[d] *C. pseudostelligera*	-	-	-

[a] Direct plunge in a Fryka Kältetechnic IMS bath set at -35°C and held for 15 and 30min., all material was thawed using a single-step warming protocol.
[b] Plunge in liquid nitrogen after 15 and 30min. at -35°C.
[c] Plunge in liquid nitrogen, with no pre-cooling step.
[d] Cryoprotectant, 5% (v/v) DMSO added at RT
[e] Cryoprotectant, 10% (v/v) methanol added at RT
[f] Cryoprotectant, 5% (v/v) DMSO or 10% (v/v) methanol added at RT
+ Post-treatment viability
- No viable cells post-treatment

3.3.1.2 *Haematococcus pluvialis*

Motile *H. pluvialis* cells were successfully cryopreserved using a two-step uncontrolled rate cooling method with post-treatment viability levels determined by employing a plate count technique (Fig. 3.1). Higher post-thaw viability (86% ± 12% in comparison to 40% ± 10%) was achieved by increasing the incubation period at -35°C to 30 min., prior to plunging into LN (Fig. 3.1). All cells were thawed using a simple single-step protocol.

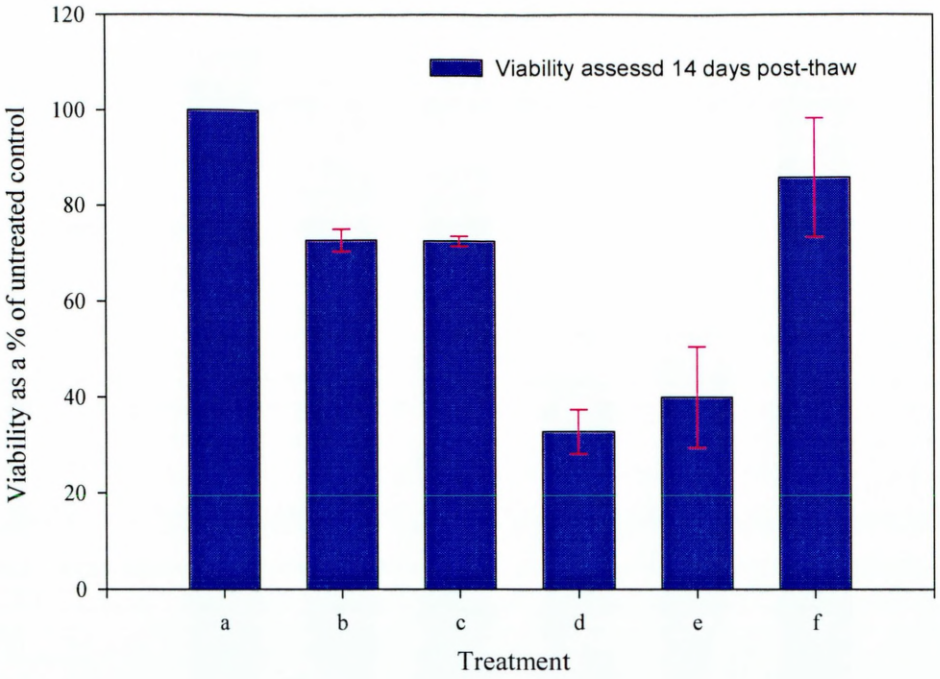


Figure 3.1 Effect of uncontrolled rate two-step cooling treatments on motile stage *Haematococcus pluvialis* cells.

(a) Untreated control, (b) Cells exposed to 5% (v/v) DMSO for 15min., (c) Cells plunged in -35°C IMS bath and held for 15min., (d) Cells plunged in -35°C IMS bath and held for 30min., (e) Cells from -35°C , 15min. exposure plunged in LN, (f) Cells from -35°C , 30 min. exposure plunged in LN.

% viability expressed as mean of the total cell population, n = 3, with errors expressed as standard errors of mean.

The application of uncontrolled rate cooling protocols to aplanospore stage *H. pluvialis* cells was less successful, as post-thaw viability was greatly reduced. The post-thaw viability for cells thawed after exposure to LN reduced from 86% ± 12% for motile cells to 5% ± 1% for aplanospore stage cells (Fig. 3.2). Furthermore, enhanced viability levels were detected in cells exposed to cryoprotectant [5% (v/v) DMSO] which were not cooled or frozen (Fig. 3.2)

The life history stage of *H. pluvialis* also influenced survival in cells cooled to the intermediate temperature. The % viability assessed against an untreated control for motile cells plunged directly into an IMS bath at -35°C for 15min. was 72% ± 1.8%, when aplanospore stage cells were treated identically, the post-thaw viability was reduced to 6% ± 0.4% (Figs. 3.1, 3.2).

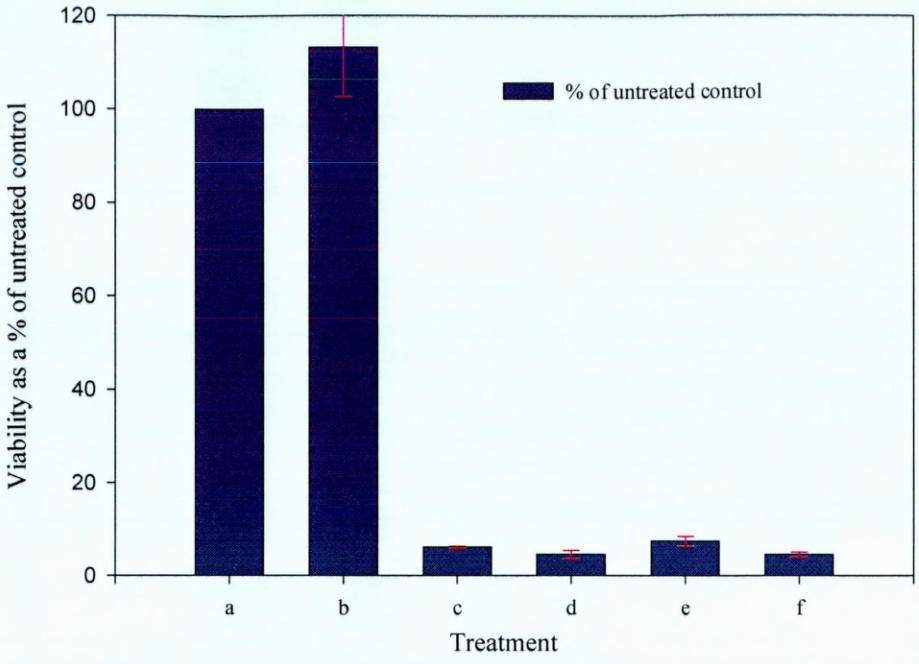


Figure 3.2 Effect of uncontrolled rate two-step cooling treatment on aplanospore stage *Haematococcus pluvialis* cells.

(a) Untreated control, (b) Cells exposed to 5% (v/v) DMSO for 15min., (c) Cells plunged in -35°C IMS bath and held for 15min., (d) Cells plunged in -35°C IMS bath and held for 30min., (e) Cells from -35°C , 15min. exposure plunged in LN, (f) Cells from -35°C , 30 min. exposure plunged in LN.

% viability expressed as mean of the total cell population, n = 3, with errors expressed as standard errors of mean.

3.3.2 Two-step controlled rate cooling

Exothermic events observed on cooling each of the cryoprotectant solutions were recorded. All solutions studied underwent a degree of supercooling before an exothermic event, attributed to heterogeneous ice nucleation, was observed. Solutions were prepared for investigation at concentrations of 5 and 10% (v/v), cryoprotectants tested were: DMSO, propylene glycol, ethylene glycol, methanol and glycerol. All solutions tested underwent supercooling to below -10°C with localised warming, in excess of 6°C, due to the exotherm associated with ice nucleation. Exotherms for the key cryoprotectant solutions employed in this study: 5% (v/v) DMSO, were initiated at -13°C ± 0.9°C with localised warming of 12°C ± 0.5°C; 10% (v/v) methanol, were initiated at -14°C ± 1.5°C with localised warming of 7.3°C ± 0.2°C.

Table 3.2 Summary of the effect of two-step controlled cooling on the post-thaw viability of selected organisms

Organism	Initial Level [a]		Current Level [b]			
	IT [c]	LN [d]	0°C [e]	-10°C [f]	IT [c]	LN [d]
E. gracilis	0	0	100	100	60	40
H. pluvialis	80	80	100	100	80	80
V. sessilis	0	0	100	100	0	0
M. aeruginosa	50	25	100	ND	60	50
C. pseudostelligera	0	0	25	ND	ND	ND
E. intestinalis	ND	ND	100	100	100	100

[a] Minimum level of post-thaw viability (% of untreated control) at the initiation (after first freeze/thaw) of the research program [*]
[b] Minimum current post-thaw viability (% of untreated control) levels achieved consistently over multiple (min. 3) freeze/thaws.
[c] Recovered from the intermediate temperature
[d] Recovered from LN
[e] Recovered from 0°C
[f] Recovered from -10°C
[*] Data from initial freezing trials, archive data within CCAP reports @ 30% post-thaw viability in *E. gracilis*.
ND Not determined

For a number of the algae studied, viable cells were recovered post-thaw using programmed controlled rate freezing protocols (Table 3.2). Optimisation and improvement of existing protocols resulted in enhanced post-thaw levels of viability in many of the algae investigated (Table 3.2)

3.3.2.1 *Euglena gracilis*

Preliminary results indicated that *E. gracilis* was freeze recalcitrant on application of the protocol detailed by Morris & Canning (1978): cells were cooled at -0.3°C $min.^{-1}$ to

-60°C, from room temperature, in cryoprotectant (10% (v/v) methanol) (Table 3.2). Following the studies detailed in this thesis a successful protocol for the cryopreservation of *E. gracilis* was achieved via a series of developmental steps.

Table 3.3 Effect of various cryoprotectants applied at different temperatures, on post-exposure viability in *Euglena gracilis*

	Temperature of manipulation				
Cryoprotectant [a]	RT [b]	0°C [c]	-30°C [d]	-60°C [e]	LN[f]
EG:JM[g]	100	100	0	0	0
DMSO	0	0	0	0	0
Propylene glycol	100	100	50	0	0
Methanol	100	100	50	50	0

[a] Cryoprotectant in appropriate media prepared at 10% (v/v)
[b] 15min. exposure at room temperature (22°C)
[c] 15min. exposure at 0°C
[d] Control cooling at -0.3°C $min.^{-1}$ to -30°C, held for 30min.
[e] Control cooling at -0.3°C $min.^{-1}$ to -30°C, held for 30min.
[f] Direct plunge in LN from -60°C
[g] EG:JM media, no cryoprotectant present
100 No significant effect on viability
50 > 50% viable cells
0 No viable cells

Based on data from single step warming protocols, n = 3.

Initial developments targeted the effectiveness of the cryoprotectants in mitigating cryoinjury and the effect of cryoprotectant toxicity on post-treatment recovery of *E. gracilis* (Tables 3.3, 3.4). It was experimentally determined that *E. gracilis* could be cooled to 0°C in the absence of cryoprotectant with no significant reduction in viability (Tables 3.2, 3.3). By pre-cooling cells to 0°C, then following a protocol which employed a single step direct addition of cryoprotectant, a 15 min. exposure period at 0°C, cooling at -0.3°C $min.^{-1}$ to -60°C, a direct plunge into LN and a single-step thawing protocol from LN or -60°C viable cells could be recovery from -60°C (> 40%) (Table

3.3). However, no viable cells were recovered after exposure to LN temperatures (Table 3.3).

Trials on different cryoprotectant solutions for cryoprotectant toxicity and effectiveness in preventing lethal injury at subzero temperatures confirmed that methanol, the preferred cryoprotectant of Morris & Canning (1978), was most effective in limiting freezing injury in *E. gracilis* (Table 3.3). However, studies on the cryoprotectant toxicity of methanol indicated that the optimum concentration of methanol was 10% (v/v) (Table 3.4).

Table 3.4 Effect of methanol concentration on post-exposure viability in *Euglena gracilis*

Concentration [a]	Time of exposure [b]					
	5	10	15	20	25	30
5	100	100	100	100	50	50
10	100	100	100	50	50	50
15	0	0	0	0	0	0

[a] Methanol concentration % (v/v)
[b] Exposure time in minutes at 0°C
100 No significant effect on viability
50 > 50% viable cells
0 No viable cells

n = 3.

Using a modified protocol; pre-cooling to 0°C followed by cryoprotectant addition and cooling at a rate of -0.5°Cmin.$^{-1}$ to -60°C, viable cells were recovered from subzero temperatures. Initial improvements in the protocol permitted enhanced recovery of viable cells from -60°C (> 50%). However, initial viability levels from LN were > 6% when cells were thawed employing a single-step thawing protocol and viability was assessed by colony development on agar. Preliminary flow cytometry viability

assessments on cells thawed using a single step thawing protocol from -60°C and LN gave post-thaw viability levels consistent with levels obtained using cell count methods (Fig. 3.3). The viability of *E. gracilis* cells recovered after exposure to -60°C and LN was 74% ± 1.6% and 4.6% ± 1.1% respectively (Fig. 3.3).

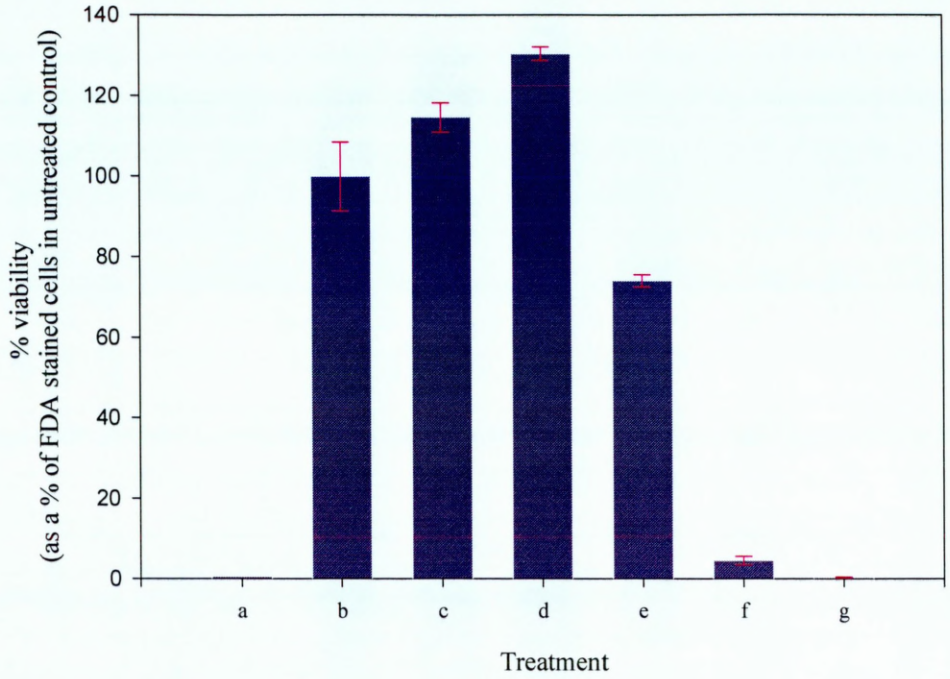


Figure 3.3 Flow cytometer viability assessments of *Euglena gracilis* exposed to different steps in a cryopreservation protocol.

(a) Unstained untreated control, (b) Untreated control, (c) Exposed to cryoprotectant at 0°C (10 % (v/v) methanol) for 15 min., (d) Held at 0°C for 15 min., no cryoprotectant, (e) Cooled at -0.5°C $min.^{-1}$ to -60°C and held for 30min., (f) Plunged into LN from -60°C, (g) Plunged directly into LN, no cryoprotectant present. Cells were thawed using a single-step protocol.

% viability expressed as mean of FDA stained, untreated control, recorded 48 h. post-thaw, of 3 replicate samples, errors are expressed as standard errors of mean.

From flow cytometry studies utilising FDA staining it was noted that a lag phase existed during which it was possible to stain cells with FDA which were almost certainly non-viable (Fig. 3.4). Following a 24 h. period of post-thaw recovery, cells were still able to demonstrate positive FDA staining in samples plunged directly into LN (Fig. 3.4). However, no viable FDA stained cells were detected after a single-step direct plunge into LN during post-thaw haemocytometer counts. After 48 h. post-thaw no positively

FDA stained cells were detected in samples grossly damaged by a single-step direct plunge into LN (Fig. 3.4).

Studies using an oxygen electrode have indicated that there may be a small reduction in photosynthetic capacity associated with exposure to the cryoprotectant solution (Fig. 3.5). In addition, in cells cooled to -60°C their photosynthetic capacity was greatly reduced, with a further reduction in photosynthetic capacity on exposure to LN (Fig. 3.5).

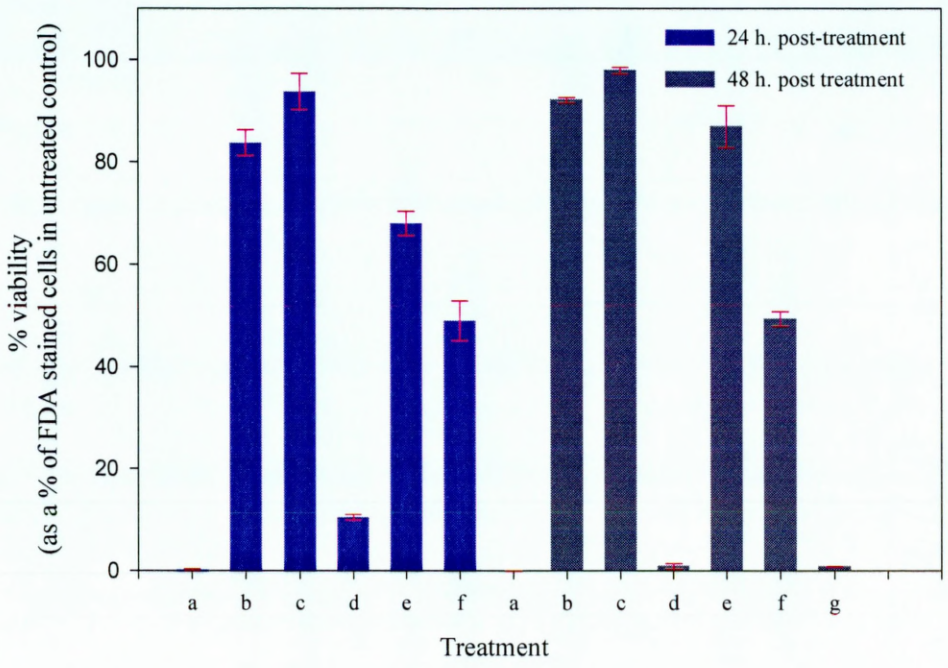


Figure 3.4 Effect of a two-step controlled rate cryopreservation protocol on the post-treatment viability of *Euglena gracilis*.

(a) Unstained untreated control, (b) stained untreated control, (c) Cells exposed to cryoprotectant at 0°C (10 % (v/v) methanol) for 15 min., (d) Cells plunged directly into LN, without cryoprotectant, (e) Cells control cooled at -0.5°C $min.^{-1}$ to -60°C and held for 30min., (f) Cells plunged into LN from -60°C, (g) Unstained, cells, plunged directly into LN, no cryoprotectant present. Cells were thawed using a two-step procedure.

% viability expressed as mean of FDA stained, untreated control, recorded 24 h. and 48 h. post-thaw, of 3 replicate samples, errors are expressed as standard errors of mean.

E. gracilis cells lose metabolic capacity after a single-step direct plunge into LN and no photosynthetic oxygen evolution was detected (Figs. 3.4, 3.5). This was consistent with the results of cell count viability assays (agar and liquid medium recovery and FDA

staining) which indicated that a single-step direct plunge into LN was lethal for *E. gracilis* (Fig. 3.3).

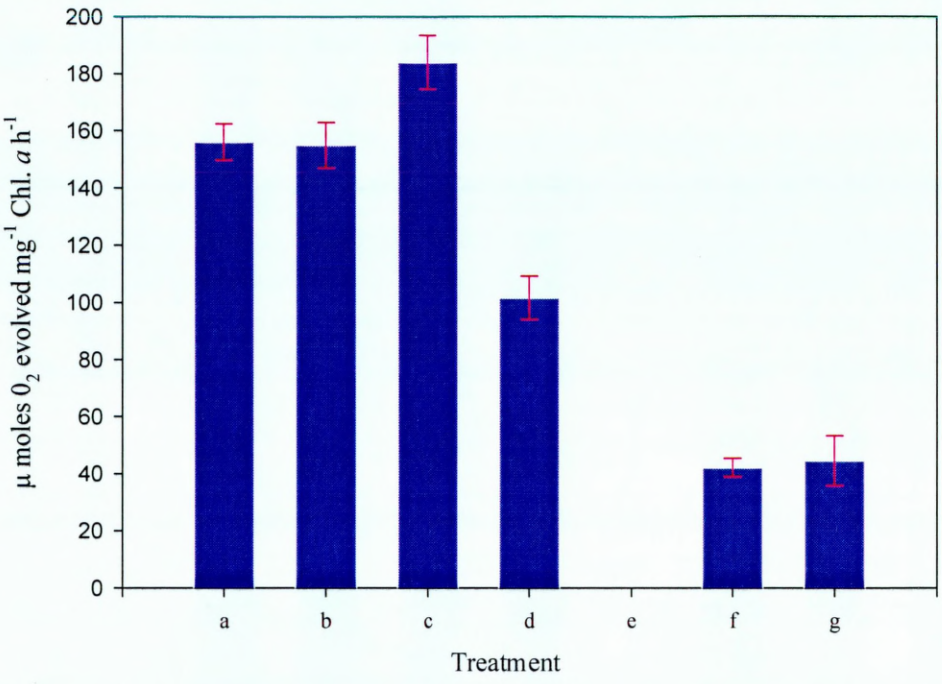


Figure 3.5 Photosynthetic activity of *Euglena gracilis* as determined after exposure to different stages of a two-step controlled cooling protocol.

(a) Untreated control cells, (b) Cells coled to 0°C and held for 15 min., (c) Removed from cryoprotectant (10% (v/v) methanol) after 15 min. exposure at 0°C, (d) Cells exposed to cryoprotectant for 15min. at 0°C, (e) Cells plunged directly into LN, no cryoprotectant present, (f) Cells cooled from 0°C at -0.5°C $min.^{-1}$ to -60°C and held for 30min., (g) Cells plunged into LN from -60°C. Cells were thawed using a simple two-step protocol.

n = 3, errors are expressed as standard errors of mean.

3.3.2.2 *Haematococcus pluvialis*

Following the successful application of two-step uncontrolled rate cooling procedures for the cryopreservation of motile *H. pluvialis* cultures the potential additional benefits offered by programmable cooling technology were investigated using both motile and aplanospore stage cultures (Table 3.5, 3.6). The influence of recovery medium were also investigated for *H. pluvialis* (Table 3.5).

Table 3.5 Effect of recovery medium on the post-thaw viability of motile and aplanospore *Haematococcus pluvialis* cells

Cell type	% Viability [a]	
	EG:JM [b]	JM [c]
Motile	70 ± 18.2	85 ± 12.5
Aplanospore	96 ± 9.3	38 ± 3.2

[a] % viability assessed against untreated control
[b] Recovery in EG:JM agar
[c] Recovery in JM agar

n = 3, errors are expressed as standard errors of mean.

Table 3.6 Comparison of cells available for colony formation with numbers of colonies observed

Treatment [a]	No. cells [b]	No. colonies observed [c]
Control (aplanospore)	79 ± 3.4	93 ± 11.2
Cryoprotectant (aplanospore)	79 ± 3.4	166 ± 22.5
Control (motile)	11.3 ± 2.3	11 ± 1.2
Cryoprotectant (motile)	11.3 ± 2.3	12 ± 2

[a] Untreated control cells and cells exposed to cryoprotectant [DMSO 5% (v/v)] (cells type).
[b] Based on haemocytometer cell counts.
[c] From plate count recovery data, assuming each cell is able to generate a colony.

n = 3, errors expressed as standard errors of mean.

Flow cytometry indicated high post-thaw viability levels for *H. pluvialis* as determined by positive FDA staining (> 90%). However, where aplanospore stage *H. pluvialis* cultures had previously been exposed to [DMSO 5% (v/v)] viability assessments commonly resulted in > 100% viability, when viability levels were assessed by firstly

employing haemocytometer cell counts to assess the number of cells available for colony formation and plate count viability assessments to determine the actual number of colonies formed (Table 3.6).

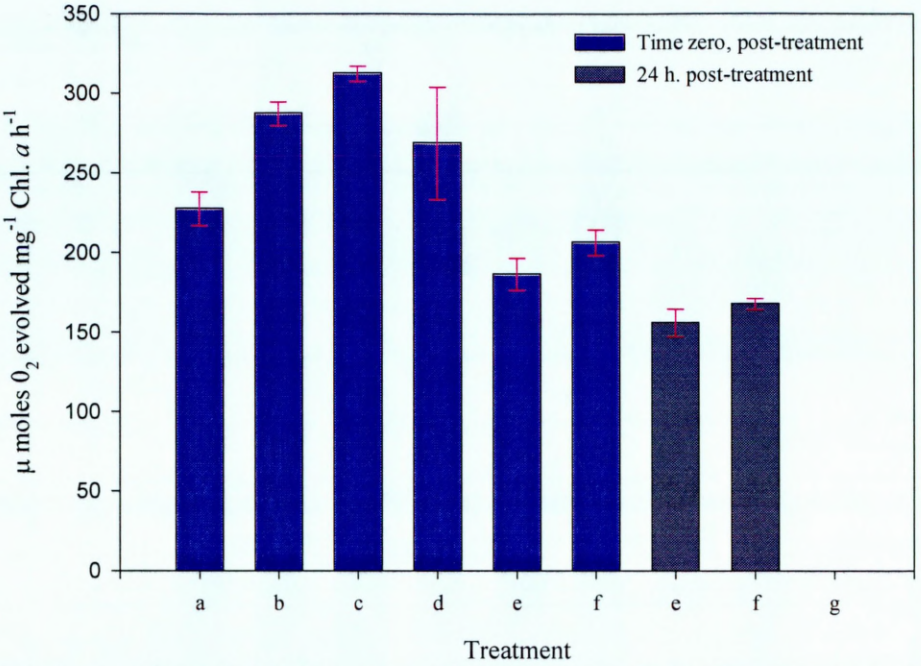


Figure 3.6 Effect of different steps of a two-step cryopreservation protocol on the photosynthetic capacity of *Haematococcus pluvialis*.

(a) Untreated control, (b) Cells cooled to 0°C and held for 20 min., (c) Cells exposed to cryoprotectant (5% (v/v) DMSO) for 15min. at RT, (d) Cells removed from cryoprotectant after 15 min. exposure at RT, (e) Cells cooled from RT at -1°C $min.^{-1}$ to -35°C and held for 30min., (f) Cells plunged into LN from -35°C, (g) Cells plunged directly into LN, without cryoprotectant. Cells were recovered using a single-step protocol.

n = 3, errors are expressed as standard errors of mean.

A comparison of viability assessment techniques was performed by comparing untreated control cells oxygen evolving capacity with cells recovered from LN after a 24 h. recovery period (91% ± 3.5%) and with flow cytometry assessments which employed positive FDA staining (94% ± 1%). These viability levels were consistent with levels obtained from plate count recovery studies, however, it was interesting to note that the deviation between replicates was greatly reduced (Fig. 3.7, Tables 3.5, 3.6).

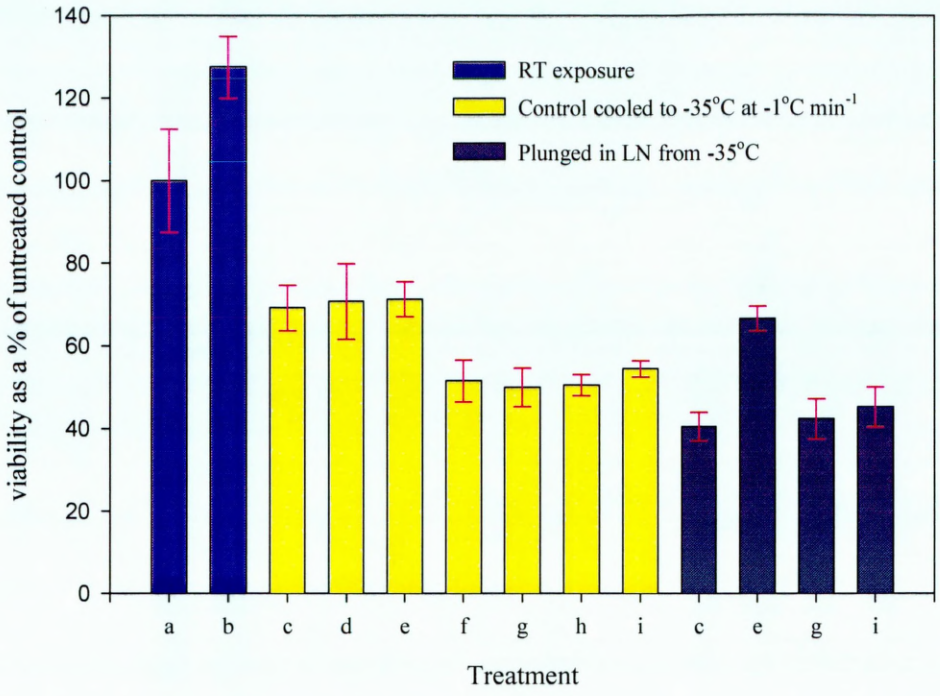


Figure 3.7 Effect of holding period at -35°C on the post-thaw viability of *Haematococcus pluvialis* aplanospore stage cells thawed after exposure to -35°C and after plunging into LN.

(a) Untreated control, (b) Cells exposed to cryoprotectant (5% (v/v) DMSO) at RT, (c) Cells held for 0 min., (d) Cells held for 10 min., (e) Cells held for 20 min., (f) Cells held for 30 min., (g) Cells held for 40 min., (h) Cells held for 50 min., (i) Cells held for 60 min.

% viability expressed as mean of total cell population, n = 3, errors are expressed as standard errors of mean.

Controlled rate cooling to different intermediate temperatures and employing different holding periods affected the post-thaw viability levels in *H. pluvialis* aplanospore stage cultures. Extending the duration of holding period at -35°C beyond 20 min., reduced post-thaw viability, on thawing from -35°C, from 70% ± 1% to 52% ± 1% (Fig. 3.7). The optimum holding period at -35°C was 20 min. (Fig. 3.7). The holding period at -60°C did not appear to have as great an effect on post-thaw viability after exposure to either -60°C or LN (Fig. 3.8). The exception to this observation was in cells exposed to LN without any holding period at -60°C (Fig. 3.8).

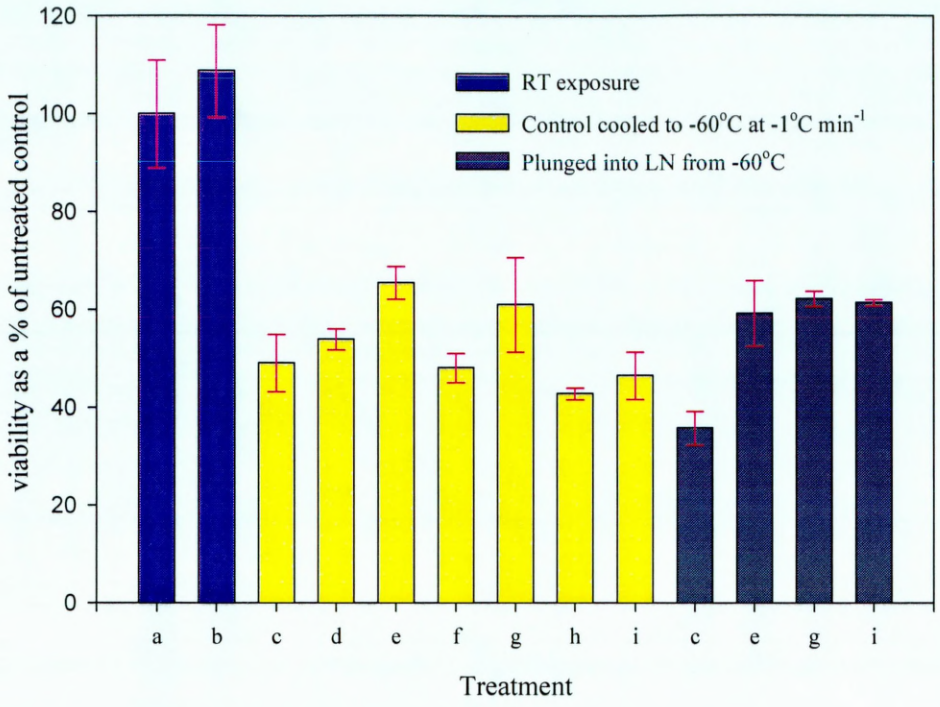


Figure 3.8 Effect of holding period at -60°C on the post-thaw viability of *Haematococcus pluvialis* aplanospore stage cells.

(a) Untreated control, (b) Cells exposed to cryoprotectant (5% (v/v) DMSO) at RT, (c) Cells held for 0 min., (d) Cells held for 10 min., (e) Cells held for 20 min., (f) Cells held for 30 min., (g) Cells held for 40 min., (h) Cells held for 50 min., (i) Cells held for 60 min.

% viability expressed as mean of total cell population, n = 3, with errors expressed as standard errors of mean.

3.3.2.3 *Vaucheria sessilis*

Studies on the effect of two-step controlled cooling protocols on *V. sessilis* initially investigated the toxicity of different cryoprotectants [methanol, DMSO, propylene glycol, ethylene glycol and glycerol at 5 and 10% (v/v)] (Table 3.7) and the effect of cooling on viability at 0°C and subzero temperatures (Table 3.8). Viability was assessed by wound healing (Fleck *et al.*, 1997a) and growth in either liquid or solid medium (see 2.5.9). *V. sessilis* responded differently to each of the cryoprotectants tested and to cooling to 0°C and -10°C in supercooled media (Tables 3.7, 3.8).

Table 3.7 Effect of exposure to cryoprotectant solution on the viability of *Vaucheria sessilis*

Cryoprotectant [a]	RT [b]	0°C [c]
DMSO 5%	+	+
DMSO 10%	+*	+*
Glycerol 5%	+	+
Glycerol 10%	-	-
Propylene glycol 5%	+	+
Propylene glycol 10%	-	-
Methanol 5%	+	+
Methanol 10%	+	+
Ethylene glycol 5%	+	+
Ethylene glycol 10%	+*	+*

[a] Cryoprotectant in appropriate media prepared at % (v/v)
[b] 15min. exposure at room temperature (22°C)
[c] 15min. exposure at 0°C
* poor recovery, with many dead filaments
+ 100% viable filaments
- 0% viable filaments

Both single and two-step thawing procedures were investigated.

Table 3.8 Effects of cryopreservation protocols and protocol steps on *Vaucheria sessilis* as assessed by cytoplasmic streaming, wound healing, filament bleaching and regrowth

Treatment	Cytoplasmic streaming	Wound healing	Bleaching of filament	Regrowth
Control	+	+	-	+
Sectioned material	+	+	-	+
22°C → 0°C	+	+	-	+
22°C → -10°C[1]	+	+	-	+
22°C → -35°C	-	-	+	-
22°C → -60°C	-	-	+	-
22°C → -60°C → -196°C	-	-	+	-
DMSO [5% (v/v)][2]	+	+	-	+
22°C → 0°C[2]	+	+	-	+
22°C → -10°C[1,2]	+	+	-	+
22°C → -35°C[2]	-	-	+	-
22°C → -60°C[2]	-	-	+	-
22°C → -60°C → -196°C[2]	-	-	+	-
Methanol [10% (v/v)][3]	+	+	-	+
22°C → 0°C[3]	+	+	-	+
22°C → -10°C[1,3]	+	+	-	+
22°C → -35°C[3]	-	-	+	-
22°C → -60°C[3]	-	-	+	-
22°C → -60°C → -196°C[3]	-	-	+	-

[1]Supercooled media/cells.
[2]Filaments incubated for 15 min. in the presence of DMSO prior to cooling.
[3]Filaments incubated for 15 min. in the presence of methanol prior to cooling.
+ 100% of filaments - 0% of filaments
Both single and two-step thawing protocols were investigated.

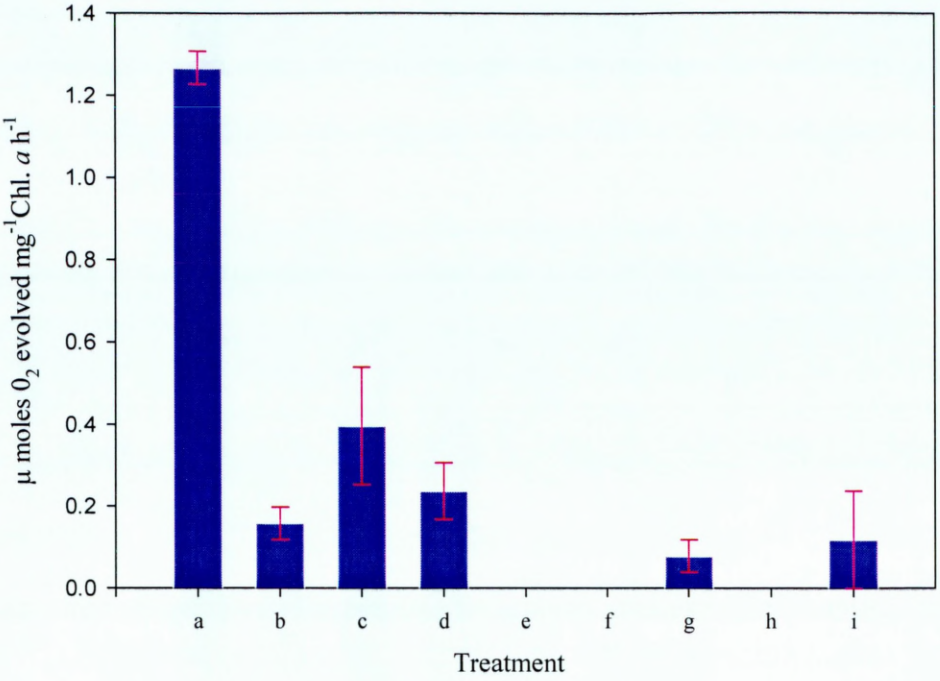


Figure 3.9 The effect of two-step cryoprotocol treatment steps on the photosynthetic capacity of the coenocytic xanthophyte *Vaucheria sessilis* immediately after thawing and 24 h. post-treatment.

(a) Untreated control, (b) Cells exposed to cryoprotectant for 15min. at 20°C, (c) Cells exposed to cryoprotectant for 15min. at 0°C (d) Cooled to 0°C and held for 15 min., no cryoprotectant present, (e) Time zero, cells cooled from 0°C at -0.5°C $min.^{-1}$ to -60°C and held for 30min., (f) Time zero, cells plunged into LN from -60°C, (g) 24 h., cells cooled from 0°C at -0.5°C $min.^{-1}$ to -60°C and held for 30min., (h) 24 h., cells plunged into LN from -60°C, (i) - Cells plunged directly into LN, without cryoprotectant. Based on a single-step thawing protocol. Cryoprotectant 10% (v/v) DMSO.

n = 3, errors are expressed as standard errors of mean.

In addition to injury caused by cooling, cryoprotectant exposure at 20°C reduced the photosynthetic capacity to 12% ± 5%, cryoprotectant exposure at 0°C also reduced the oxygen evolving capacity to 31% ± 19% (Fig. 3.9). Furthermore, exposure to 0°C in the absence of cryoprotectant reduced the oxygen evolving capacity of *V. sessilis* to 19% ± 9% (Fig. 3.9).

3.3.2.4 Other strains examined

Microcystis aeruginosa recovered after controlled cooling and exposure to LN with

high levels of viable cells (>50%). This demonstrated that it was possible to improve post-thaw viability levels attained with two-step uncontrolled rate cooling protocols through the use of controlled programmable cooler technology. The improved degree of control over cooling rate compared to direct immersion in a pre-cooled IMS bath offers greater scope for the optimisation of two-step cooling regimes. This was clearly demonstrated for *M. aeruginosa* by the higher post-thaw viability achieved through the use of controlled cooling rates (62 ± 1%), in comparison to uncontrolled rate two-step cooling (25 ± 1.5%).

Cyclotella pseudostelligera survived exposure to DMSO [5% (v/v)]. However, no survival was attained from subzero temperatures. *C. pseudostelligera* tolerated cooling to 0°C, both in the presence and absence of cryoprotectant (> 20%), however, viability was reduced on prolonged exposure to the cryoprotectant (< 8%).

Euglenophyta

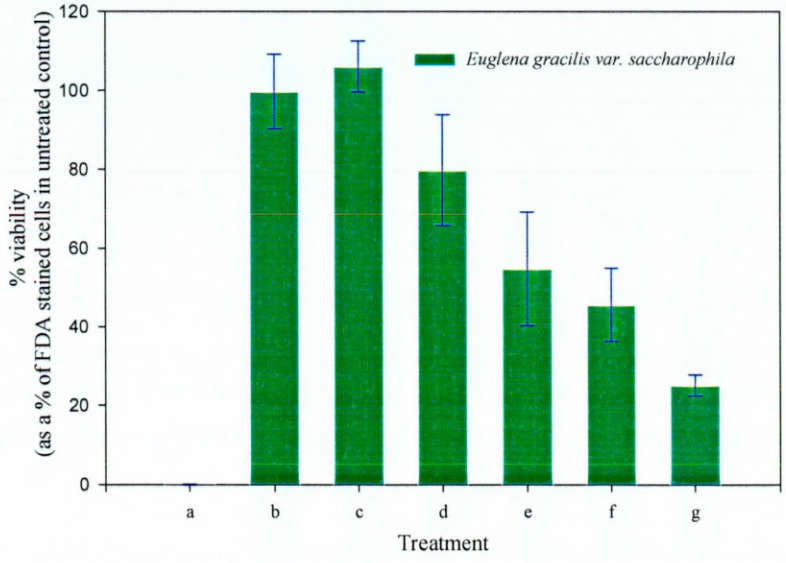


Figure 3.10 Use of flow cytometry to assess viability of the achlorophylous strain of *Euglena gracilis* var. *saccharophila.*

(a) Unstained untreated control, (b) Cells stained untreated control, (c) Cells exposed to cryoprotectant at 0°C (10 % (v/v) methanol) for 15 min., (d) Cells held at 0°C for 15 min., without cryoprotectant, (e) Cells cooled at -0.5°C $min.^{-1}$ to -60°C and held for 30min., (f) Cells plunged into LN from -60°C, (g) Cells plunged directly into LN, without cryoprotectant. Cells were thawed using a single-step protocol.

% viability expressed as mean of FDA stained, untreated control, recorded 24 h. post-thaw, of 3 replicate samples, errors are expressed as standard errors of mean.

Euglenophyta selected for their similarity to *E. gracilis* were studied to assess the suitability of the *E. gracilis* cryopreservation protocol for use on similar organisms. These studies also presented the opportunity to investigate the influence of the presence/absence of photosynthetic capacity and the presence/absence of a flagellum on the applicability of this technique to other euglenoids (Figs. 3.10-12).

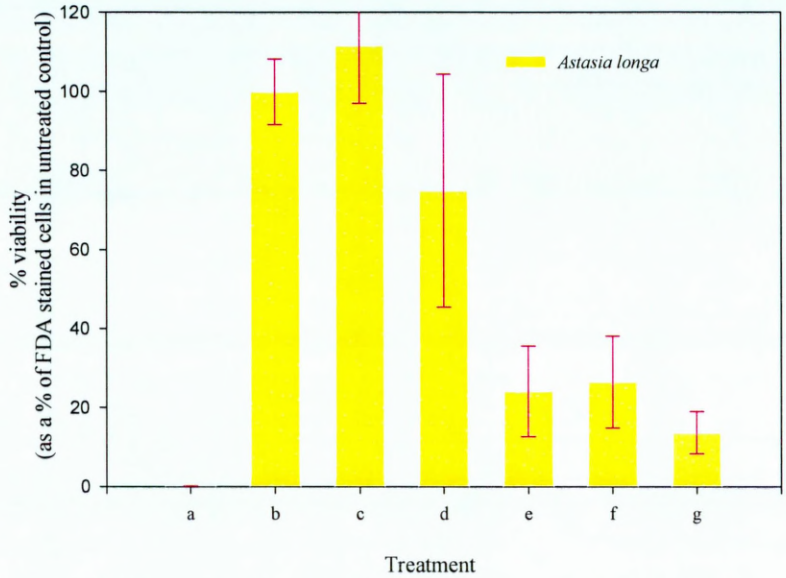


Figure 3.11 Use of flow cytometry to assess viability of *Astasia longa*.

(a) Unstained untreated control, (b) Cells stained untreated control, (c) Cells exposed to cryoprotectant at 0°C (10 % (v/v) methanol) for 15 min., (d) Cells held at 0°C for 15 min., without cryoprotectant, (e) Cells cooled at -0.5°C $min.^{-1}$ to -60°C and held for 30min., (f) Cells plunged into LN from -60°C, (g) Cells plunged directly into LN, without cryoprotectant. Cells were thawed using a single-step protocol.

% viability expressed as mean of FDA stained, untreated control, recorded 24 h. post-thaw, of 3 replicate samples, errors are expressed as standard errors of mean.

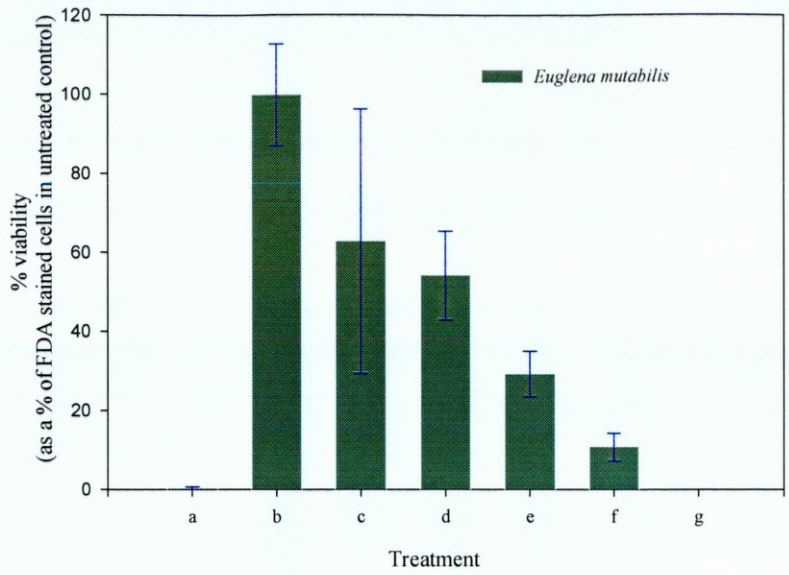


Figure 3.12 Use of flow cytometry to assess viability of *Euglena mutabilis*.

(a) Unstained untreated control, (b) Cells stained untreated control, (c) Cells exposed to cryoprotectant at 0°C (10 % (v/v) methanol) for 15 min., (d) Cells held at 0°C for 15 min., without cryoprotectant, (e) Cells cooled at -0.5°C $min.^{-1}$ to -60°C and held for 30min., (f) Cells plunged into LN from -60°C, (g) Cells plunged directly into LN, without cryoprotectant. Cells were thawed using a single-step protocol.

% viability expressed as mean of FDA stained, untreated control, recorded 24 h. post-thaw, of 3 replicate samples, errors are expressed as standard errors of mean.

Table 3.9 Post-thaw viability levels as assessed by FDA staining in a range of euglenoids after exposure to the intermediate holding temperature and LN of a two-step cryopreservation protocol.

Organism	-60°C [a]	LN [b]
Euglena gracilis [f]	74 ± 2	5 ± 1
Euglena gracilis var. *saccharophila* [f]	55 ± 14	45 ± 9
Astasia longa [f]	24 ± 11	27 ± 12
Euglena mutabilis	29 ± 6	11 ± 3

[a] Control cooled at -0.5°C $min.^{-1}$ to -60°C and held for 30°C min., thawed using a single-step protocol, viability assessed 24 h. post-thaw.
[b] Direct plunge in LN from -60°C, thawed using a single-step protocol, viability assessed 24 h. post-thaw.
[f] Flagellate

n = 3, errors are expressed as standard errors of mean.

All cultures appeared viable when observed using light microscopy up to 96 h. post-thaw. Culture recovery beyond this point was not performed.

3.3.3 Long-term storage of protists

Long-term storage trials were implemented to assess the stability of cultures after prolonged storage under LN. *H. pluvialis* was preserved using standard two-step controlled cooling protocols with an intermediate holding period of either 15 or 30 min. Cells recovered using standard procedures and optimum holding period of 15 min. gave > 70% post-thaw viability. No change in viability was detected after 12 months of cryopreservation, the intermediate holding period did not affect the long-term stability of cryopreserved cells.

3.3.4 Temperature stability in the cryostat

Minor fluctuations in temperature were observed during a three week period of monitoring temperature levels in a cryostat. Temperature fluctuations corresponded to the routine addition of liquid nitrogen and routine addition/retrieval of vials from an adjacent section of the inventory system (Fig. 3.13). However, these fluctuations were too small to be of any significance to the stored material.

Simulating removal/addition events for vials stored at the bottom, middle and top of the inventory system demonstrated that fluctuations in temperature may be experienced both by the manipulated vial and vials in other sections of the inventory (Fig. 3.14). Removal of a vial from the bottom section resulted in the temperature rising to -112°C, a temperature which was high enough to potentially be associated with problems associated with ice crystal growth and freeze-fracture events. However, the duration of exposure of specimens to the elevated temperature was extremely short.

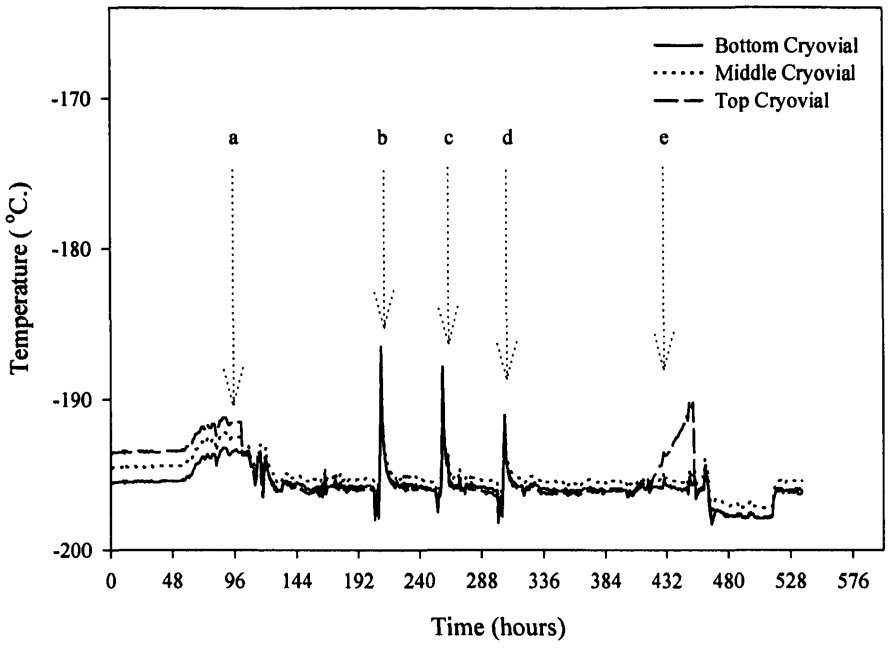


Figure 3.13 Measurement of temperature fluctuations in the CCAP cryostore over a three week time course.

Letters a, b, d and e indicate manual addition of liquid nitrogen to the cryostat, c indicates removal/addition of cryovials from/to an adjacent inventory in the cryostat.

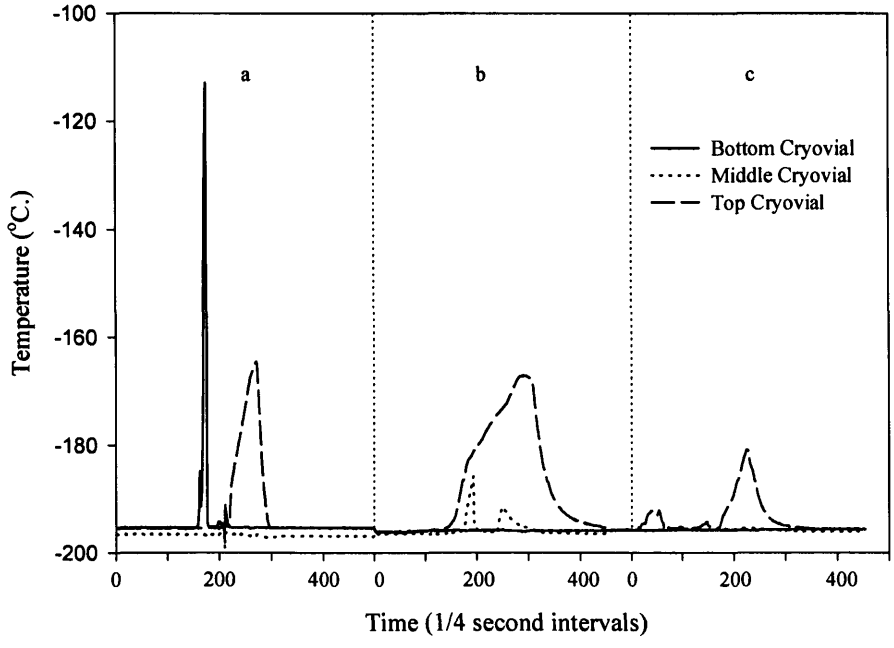


Figure 3.14 Simulation of cryovial addition/removal from the bottom, middle and top inventory locations.

Letters signify addition/removal from (a) the bottom (b) the middle and (c) the top drawers of the inventory system in the CCAP cryostore.

3.4 Discussion

Two-step cryopreservation can clearly result in high levels of post-thaw viability (Table 3.2). This is consistent with the reports of viability levels in excess of 95% in some members of the Chlorococcales (Morris, 1978). The ability to routinely attain high levels of post-thaw survival, minimises the possibility of selecting a preservation tolerant sub-population during cryopreservation. This supports the use of cryopreservation as a routine method for the long-term preservation of algal strains. Additional advantages of cryopreservation, include: the prevention of genetic change in preserved cultures and reductions in labour costs compared with traditional serial subculture techniques (Day *et al.*, 1997; Grout *et al.*, 1990). In the studies detailed above, the most successful cryoprotectant additives can be classed as penetrating. Both methanol and DMSO are able to rapidly enter cells (Meryman, 1971), however, they have also been associated with cryoprotectant toxicity in test systems (Meryman, 1971). Cryoprotectant toxicity was identified during this study, where prolonged exposure to cryoprotectants, or exposure to suboptimal cryoprotectants, resulted in cell mortality [in *C. pseudostelligera* (< 8% survival), *E. gracilis* (Tables 3.3, 3.4) and in *V. sessilis* (Table 3.7)]. In addition, cryoprotectant exposure was observed to be capable of inhibiting cellular metabolic processes in *E. gracilis* (Fig. 3.5) and in *V. sessilis* (Fig. 3.9).

During both uncontrolled rate and controlled rate two-step cooling protocols, supercooling of the liquid medium below its thermodynamic freezing point occurred, and the principles of supercooling are discussed in 1.9.2. In aqueous solutions, ice formation is usually by heterogeneous nucleation, where nucleation occurs preferentially at foreign surfaces at temperatures well above the homogeneous ice nucleation temperature of water (-38.5°C) (Hobbs, 1974; Mehl, 1996b). Exothermic studies performed using the Planer Kryo 10 Mk III, programmable cooler, demonstrated that all cell samples were able to undergo supercooling before ice nucleation occurred in the external solution. All of the cryoprotectant solutions studied underwent a degree of supercooling (to below -10°C) before an exothermic event (in excess of 6°C) was detected due to the liberation of the latent heat of fusion (Morris, 1981) (3.3.2).

Cryoprotectant solutions of 5% (v/v) DMSO and 10% (v/v) methanol were demonstrated to supercool to -13°C ± 0.9°C and -14°C ± 1.5°C respectively.

It is possible that the platinum probe located in the vial to record the exotherm acted as a suitable foreign surface at which crystallisation could be nucleated. However, the platinum nucleator was not present in vials prepared for cryopreservation, presenting the possibility that there may be variation between the ice nucleation temperature detected using the probe and that in sealed cryovials. The media, will, however, still undergo supercooling, until a suitable site for ice nucleation exists. This was confirmed by direct observation of vials selected at random and recovered from subzero temperatures. It can, therefore, be assumed that media in all cryovials, cooled to subzero temperatures, underwent a degree of supercooling, nominally to a temperature below -10°C. However, the onset of heterogeneous ice nucleation remains an unpredictable event (Taylor, 1987).

3.4.1 Application of two-step cooling to algae

In plant systems, freezing injury has previously been classified as being of three distinct types; 1) primary direct injury, due to intracellular freezing; 2) secondary freeze-induced dehydration injury, due to extracellular freezing, and 3) injury, due to other freeze-induced stresses (Levitt, 1980). During the course of this study investigating the effects of two-step cooling and freezing, a similar range of cryoinjuries were observed. In addition, studies on the photosynthetic oxygen evolving capacity of cells and flow cytometry studies employing vital viability stains have highlighted the possibility that additional lethal and non-lethal events may be occurring which may be associated with cellular metabolic pathways. The observation that the cell's oxygen evolving capacity may be affected by exposure to low temperatures and cryoprotectant solution indicates a possible biochemical mode of cryoinjury and the potential for cryoprotectants to influence the alga's metabolic capacity as well as providing protection from cryopreservation stresses (Fig. 3.5, 3.6). In addition, studies employing flow cytometry, demonstrated that enzyme activity may continue, even in lethally damaged cells presenting the possibility that sites of cryoinjury, other than membrane rupture may exist (Figs. 3.4, 3.10, 3.11).

Cryoprotectant toxicity and the period of exposure to increased solute concentrations may be a significant cause of freezing injury and subsequent death (Pegg, 1987) and have previously been categorised as secondary freeze-induced injury and cryoprotectant toxicity. In studies by Morris (1981) increasing molarity of NaCl in unfrozen water at various subzero temperatures was monitored, in water lacking cryoprotectant the molarity in the unfrozen solution increased from 0.15 M to 5 M by -30°C, however, in water containing 3.0 M glycerol the molarity of the unfrozen solution plateaued at < 1 M). During two-step cryopreservation protocols, cells are subjected to osmotic changes with potentially damaging increases in extracellular solute concentration during freezing. If not fully mitigated by cryoprotectants, the resulting rise in solute concentration may potentially damage the organism. In the experimental systems this was most commonly demonstrated by reduced viability levels in cells cooled at slower rates or exposed for extended periods to cryodehydration (3.3.2.1, Fig. 3.7). In studies employing *M. aeruginosa*, viability was reduced from 62% ± 2% to 50% ± 1.5% when the intermediate holding period was extended by 15 min., *H. pluvialis* was also demonstrated to be susceptible to the duration of exposure to the intermediate holding temperature at -35°C (Fig. 3.7).

Experimental optimisation of the cryoprotectant application concentration and duration of exposure were necessary for the development of optimum protocols (Tables 3.3, 3.4, 3.7, 3.8). *E. gracilis* was particularly sensitive to exposure to methanol at concentrations of 15% (v/v) and above, and to prolonged exposure periods at the optimum concentration of methanol [10% (v/v)] (Table 3.4). *V. sessilis* also exhibited varying responses to different cryoprotectants and cryoprotectant concentrations (Table 3.7). Selection of a suboptimal cryoprotectant resulted in reduced cryoprotection or lethal toxic injury in *E. gracilis* (Table 3.3) The addition of cryoprotectant is, however, necessary because it acts to mitigate injury encountered during freeze-induced dehydration (Steponkus *et al.*, 1992). Furthermore, it protects cells by modulating rises in solute concentration during freezing (Pegg, 1987). During successful two-step cryopreservation, concentration of the remaining solution eventually reaches a point at which it forms a glass so preventing any further concentration of the supercooled cytosol (Steponkus *et al.*, 1992). This would occur if sufficient time was allowed for

osmotic equilibrium to take place, *i.e.* in slowly cooled specimens. The hypothesis which may be drawn, that an ever slower cooling rate need only be adopted to secure successful cryopreservation, does not hold true, and was demonstrated by a reduction in post-thaw viability levels when *E. gracilis* was cooled at the slower cooling rate of -0.3°C min^{-1} (3.3.2.1) (Fleck *et al.*, 1996; 1997b; reviewed by Mazur, 1970).

During two-step cooling the external solution will freeze resulting in an ever decreasing unfrozen proportion and an associated rise in the osmotic potential across the cell membrane. This change in the chemical potential of the water in the external medium will cause the cell to loose water across the cells semi-permeable membrane (Toner, 1993). During uncontrolled two-step cooling, the change in the osmotic potential across the cell membrane, driven by the rapid freezing of the external solution, is likely to be rapid and potentially more injurious than in controlled rate cooling. Slow controlled rate cooling would result in more gradual freezing and a slower change in the osmotic potential across the cell membrane and potentially less variation in the point of heterogeneous ice nucleation.

Unsuccessful preservation using two-step cooling may be due to the inability of the cryopreservation protocol to effect sufficient cellular dehydration and concentration of the cytosol, in order to prevent intracellular ice crystallisation (Chapter 4). In rapidly cooled cells, the period during which concentration of the cytosol can occur is reduced, promoting intracellular ice nucleation at higher subzero temperatures. Slower cooling rates depress the intracellular ice nucleation point further (Toner, 1993). This effect is likely to be detected under experimental conditions where slower cooling rates or prolonged periods of cryodehydration are employed. This may allow viable cells to be recovered post-thaw, or increase levels of post-thaw viability (Figs. 3.2, 3.7). The post-thaw viability of *H. pluvialis* aplanospore cells was influenced by the period of cryodehydration and the rate of cooling employed. Uncontrolled two-step cooling was more rapid than the controlled rate cooling protocol (-1°C min^{-1}) employed for *H. pluvialis*, and resulted in post-thaw viability levels of 6% ± 0.4% in comparison to 70% ± 1% attained using slower controlled cooling rates.

Post-nucleation, ice morphology and crystal dimensions are influenced by the degree of supercooling and the rate of cooling (Taylor, 1987). In slowly cooled water, ice crystals form around the limited number of early-developing nuclei and as the temperature falls these crystals grow larger rather than form new crystals at new generating sites (Taylor, 1987). This phenomenon may be of importance where protocols employ different cooling regimes, with the potential for the temperature of heterogeneous ice nucleation to vary by a greater degree in the more rapidly cooled uncontrolled rate cooling protocols. This introduces the possibility of wider variations in the point of heterogeneous ice nucleation between different freezing runs and between vials. This, in turn, causes wide variation in the thermal histories of frozen materials. Wide variations in the thermal histories, to which cells within a single freezing run are subjected, could influence post-thaw viability levels due to the unpredictable nature of heterogeneous ice nucleation and may lead to large deviations in post-thaw viability levels between vials and cryopreserved batches of the same strain.

Studies on the organisms investigated have clearly demonstrated wide variations in the responses of different organisms to cryopreservation. In this study the prokaryotic *M. aeruginosa* proved readily amenable to cryopreservation. This was not altogether unexpected because prokaryotic cyanobacteria are closely related to other non-photosynthetic bacteria which have historically proven readily amenable to cryopreservation (Hasegawa, 1994). The eukaryotic *H. pluvialis* also proved readily amenable to cryopreservation. The fact that *H. pluvialis* is regularly exposed to dehydration, temperature and illumination stress in its natural environment and posses adaptations for surviving these stresses (Harker *et al.*, 1996), may contribute to the ease of cryopreserving this organism. In addition, the relatively complex eukaryotic macroalga *Enteromorpha intestinalis* was also successfully cryopreserved. This organism is also commonly exposed to stresses associated with desiccation, wide temperature variations and salinity changes (osmotic stress) in its natural environment, the upper littoral zone of the intertidal environment. Adaptations to dealing with desiccation, osmotic and temperature stresses in an organisms natural environment may therefore help it to overcome the stresses encountered during cryopreservation. It is also worth noting that the ability of an organism to successfully survive a two-step cooling protocol can also be considered as being dependant upon its ability to withstand slow

cooling to subzero temperatures, with many examples of chilling intolerant organisms existing (Levitt, 1980) (1.9.1). All the strains investigated in this study were able to tolerate cooling to 0°C (Table 3.2) demonstrating that they were not simply chill sensitive.

3.4.2 *Haematococcus pluvialis* and *Microcystis aeruginosa*

Both *H. pluvialis* and *M. aeruginosa* were successfully cryopreserved using a uncontrolled rate two-step cooling regime (Table 3.1). Viability levels for *H. pluvialis* were in excess of 80% (Fig. 3.1) and *M. aeruginosa* was recovered from LN with > 20% viability. The long-term preservation of *M. aeruginosa* solely by cryopreservation would not be practical using this uncontrolled rate cooling, two-step, cryopreservation protocol, as the post-thaw viability levels were not high enough to preclude the possibility of selection of a tolerant sub-population. In comparison, the high post-thaw viability levels attained for *H. pluvialis* would be considered high enough to allow preservation by cryopreservation with minimal possibility of selection of sub-populations. However, studies examining the effect of two-step uncontrolled cooling on distinct life history stages of *H. pluvialis* indicated that significant variations in post-thaw viability levels may occur. Motile stage cells were recovered with > 80% viability whereas aplanospore stage cells only achieved 5% post-thaw viability (Figs. 3.1, 3.2). It is possible that the differences in the structure of the aplanospore stage cells from the motile stage cells contributed to their lower post-thaw viability levels. Aplanospore stage cells are adapted to withstand the effects of dehydration in their natural environment, this may make them more difficult to cryodehydrate than the motile cells. The consequence of this is that on cooling, cells experienced lethal intracellular ice formation (see Chapter 4). In addition, the relatively impermeable cell wall of the aplanospore stage cells may restrict penetration by the cryoprotectant (DMSO). However, DMSO is a rapidly penetrating cryoprotectant and would be expected to be able to readily enter aplanospore stage cells, making insufficient cryodehydration a more likely cause of cryoinjury (Meryman, 1971). Rapid cooling, together with reduced cryodehydration, in two-step direct cooled aplanospore stage cells was likely to result in intracellular ice nucleation at high subzero temperatures. This phenomenon was considered to be a primary cause of lethal injury in this cell type.

For the practical application of cryopreservation within a culture collection, cryopreservation protocols employed must be robust and give reproducible levels of post-thaw viability. The wide variations in post-thaw viability demonstrated for *H. pluvialis* which were dependant upon the algas' life history stage could make routine application of this preservation technique inappropriate. A robust preservation protocol would require that only motile stage cells were present, as cultures with high levels of aplanospores could potentially result in the selection of a freeze tolerant sub-population. An additional factor worth consideration, is that motile stage cultures are the youngest cultures and tend to have a lower cell density. In a working culture collection where cultures would be recovered for an order, low cell density in cryopreserved material would greatly increase the period between order and delivery. In the case if *H. pluvialis* an inoculation of 1ml (1.5×10^4 cells) in 50ml of EG:JM media would take over two weeks to give a cell density high enough for dispatch.

Using programmable coolers, both motile and aplanospore stage cells of *H. pluvialis* were cryopreserved with high levels of post-thaw viability (Table 3.5). Benefits were also observed for *M. aeruginosa* whose post-thaw viability was raised from 25% ± 1% to 62% ± 1%. The slower cooling rate from room temperature to -35°C permitted an increased exposure time to the cryoprotectant and for cryodehydration of the cell in the frozen environment. Aplanospores required a much longer period of exposure to the cryoprotectant, in comparison to motile cells. Their thick cell walls, are relatively impermeable and possibly slow permeation of the cryoprotectant. However, DMSO readily crosses cellular membranes conferring cryoprotection by colligatively modifying the composition of the solution at subzero temperatures (McGann *et al.*, 1987). Differences in viability, of aplanospore stage *H. pluvialis* cells, obtained between the direct cooling (5%) and the controlled cooling (96%) protocols, may be explained by an increase in the time available for the DMSO to penetrate the cell and a greater period of time available for cryodehydration, at temperatures above the point where intracellular ice is nucleated. The effective exposure time to cryoprotectant at temperatures above 0°C was increased by 25 min., however, extended exposure to DMSO at temperatures above 0°C may also result in cell damage (Matthes & Hackensellner, 1981). The observation that the same protocol also permits high viability levels to be attained with

motile cell, indicated that *H. pluvialis* cells were highly tolerant of the effects of dehydration and cryoprotectant exposure.

During successful two-step cryopreservation, concentration of the intracellular solution would eventually reach a point at which it forms a glass, preventing any further concentration of the supercooled cytosol (Steponkus *et al.*, 1992). This would occur if sufficient time was allowed for osmotic equilibrium to take place, permitting cryodehydration to progress to a point where the cytosol vitrifies in preference to homogeneous ice nucleation (Steponkus *et al.*, 1992). This goes some way to explaining the benefit gained from prolonged holding periods at the intermediate temperature. However, cryoprotectant toxicity and the period of exposure to increased solute concentrations may also be a significant cause lethal injury (Pegg, 1987). Prolonged incubation at -35°C (> 20 min.) reduced levels of post-thaw viability in *H. pluvialis* thawed from -35°C and may be attributed to secondary freeze-induced dehydration injury (Levitt, 1980). If cells were then plunged into LN after an insufficient period for cryodehydration at -35°C viability levels post-thaw were also reduced and could be attributed to primary direct injury due to intracellular freezing (Levitt, 1980) (Fig. 3.7).

However, when cooling was continued to -60°C, followed by a holding step at -60°C, no further reduction in viability was observed (Figs. 3.7, 3.8). It was likely that at -35°C damage occurred to the cell due to: excessive cryodehydration, intracellular ice-crystal growth or changes to osmotic potential across the cell membrane as described by Levitt (1980). At the lower temperature (-60°C), the apparent lack of damage observed in the system, may be due to vitrification of the cells occurring between -35°C and -60°C. Alternatively, physical phenomenon due to the dependence of osmotic pressure on the rate of molecular motion, so that a decrease in the rate of the process is proportional to the fractional change in absolute temperature, would reduce the amount of dehydration experienced in the cells at -60°C and may have prevented them from reaching their critical dehydration limit (Taylor, 1987).

The study on *H. pluvialis* revealed a number of interesting effects of cryotreatment on the cells. Enhanced recovery and oxygen evolving capacity were observed in cells

treated with DMSO [5% (v/v)]. This observation may be due to the influence of DMSO on the cells metabolic capacity. In addition, enhanced levels of viability were observed when aplanospore stage cells were recovered after treatment with DMSO. This was attributed to DMSO promoting cell division, resulting in the release of large numbers of daughter cells (*H. pluvialis* can produce up to 32 daughter cells from a single cell, personal observation). Large numbers of daughter cells have also been reported in studies on encysted *Haematococcus* spp. (Lee & Ding, 1994). In addition, DMSO has been previously used to initiate cell division in arrested callus protoplasts (Hahne & Hoffmann, 1984) and has been linked to increased growth rates in bean plants, the fungi *Penicillum notatum* and *Aspergillus niger* (Beauchamp & Crete, 1968; Herschler, 1968). This ability to promote cell division may be responsible for the increased levels of cell numbers in cultures exposed to the cryoprotectant. It is important to fully understand the action of DMSO on *H. pluvialis* cell, particularly the aplanospore stage cells. If cell division may be enhanced in cells exposed to DMSO, it is possible that the post-thaw viability levels may have been artificially enhanced. This may explain differences in viability levels obtained using haemocytometer counts of vital stained cells and viability levels determined using colony generation in agar plates of 79% ± 3.4% to 166% ± 22.5% respectively.

Improved recovery, particularly of the aplanospore stage cells on EG:JM media (Table 3.5) indicated that there may be benefits from recovery on nutrient rich media, this has previously been observed for other algae (Morris, 1976b; Day & DeVille 1995). In studies employing flow cytometry and oxygen evolution measurements on motile *H. pluvialis* cells, the variation between levels of viability in each of the triplicate readings was relatively small. Reduced variation between replicates, was detected, when viability levels were determined using methods other than colony generation in agar plates. The large variations between replicate agar plates used in colony generation viability assessments, may be attributed to insufficient mixing of cells in the agar, loss of cells due to heat shock on addition of the molten agar, or clumping of cells due to stresses encountered during cryopreservation.

3.4.3 Euglenophyta

The effective use of programmable cooling technology was initially limited by cryoprotectant toxicity and the period of exposure to the cryoprotectant at temperatures above freezing (Table 3.3). By freezing from 0°C at -0.3°C $min.^{-1}$ viable cells (> 40%) were obtained from -60°C. However, no viable cells were obtained from LN (Table 3.3). The use of a faster cooling rate -0.5°C $min.^{-1}$ to -60°C allowed viable cells to be recovered from both -60°C and LN with viability levels of 74% ± 2% and 5% ± 2% respectively. This was assumed to be due to excessive dehydration occurring at the slower cooling rate, exposing the cells to excessive dehydration stress and ultimately lethal injury (Levitt, 1980) (1.9.2-1.9.4, Chapter 5). By employing a slightly faster cooling regime, cells were able to undergo sufficient cryodehydration to prevent intracellular ice formation, yet were not subjected to lethal levels of dehydration stress (Fig. 3.3).

Viability assays 24 h. and 48 h. post-thaw demonstrated that some intact, but non-viable cells are able to undergo FDA staining up to 24 h. post-thaw (Fig. 3.4). These cells must have active enzymes and an intact cell membrane. However, the cells were non-viable, or were unable to recover from the stresses to which they have been exposed during the cryopreservation protocol. In this study and in previous reports algae have been demonstrated to able to give false FDA reading up to 24 h. post-thaw (Fenwick & Day, 1992; Day & Fenwick, 1993). These cells may have membrane damage due to oxidative stress which although lethal had not resulted in membrane rupture, additionally lethal damage may have occurred intracellularly. Photosynthesis/respiration investigations carried out with an oxygen electrode have also demonstrated that the photosynthetic capacity of the cells can be reduced by exposure to low-temperatures and/or cryoprotectant exposure, indicating that the cryopreservation protocol may effect the photosynthetic ability of the cells, this may indicate that chloroplasts are damaged by the cryopreservation protocol (Fig. 3.5) (Chapter 4). In addition, this indicated that this organism was sensitive to both the cryoprotectant and reduced temperatures suggesting that injury may be due to biochemical events (Chapter 7).

The importance of both cooling and warming rate on cell survival has previously been described for cryopreserved cells (Jacobsen *et al.*, 1984; Pegg *et al.*, 1997). The optimum warming rate in frozen rabbit kidneys was found to be 1°C min^{-1} (Jacobsen *et al.*, 1984). However, in material recovered from cryostorage temperatures cell injury resulted on slow warming rates, in which cell damage was demonstrated to correlate positively with intracellular ice (Karlsson *et al.*, 1993). In control cooled elastic arteries rapid warming resulted in gross fractures (Pegg *et al.*, 1997). It has been suggested that the fractures probably resulted from thermal stresses created by rapid warming of the vitreous material that was produced by freeze-concentration of the aqueous phase (Pegg *et al.*, 1997). By applying relatively slow warming to -100°C, at which temperature the vitreous material has softened, the stresses encountered are reduced and fractures avoided (Pegg *et al.*, 1997). These findings clearly indicate the importance of achieving an optimum warming rate. Material can almost certainly be warmed to temperatures below which ice crystal growth can occur without any loss of viability (Morris, 1981). However, if further warming occurs the probability of ice crystal growth increases and with it the possibility of primary direct freeze induced injury. Further improvements in the post-thaw viability of *E. gracilis* was achieved through the adoption of a two-step thawing protocol. Two-step warming was able to increase post-thaw viability levels in *E. gracilis* (Table 3.2, Fig. 3.4). The two-step thawing procedure adopted a 1 min. holding period in air prior to plunging into a pre-heated waterbath at 40°C. The increase in post-thaw viability may be explained by the relaxation of the intracellular vitreous state, avoiding lethal freeze fracture events during the slow warming in air, followed by rapid warming in the waterbath which was able limit, or prevent, ice crystal growth intracellularly (Figs. 3.3-3.5).

In studies on strains with similar morphologies to *E. gracilis*, the lag phase between post-thaw recovery and a true representation of cell death was observed in *Astasia longa* and *E. gracilis* var. *saccharophila* but not in *E. mutabilis* (Figs. 3.10-3.12). Organisms which had a detectable "false positive" FDA stain up to 24 h. post-thaw were those organisms which were flagellate. It is possible that the "false positive" may be due, in part, to the increased mitochondrial content of these cells connected with flagellar function. Viability post-thaw did not appear to be affected by the presence or absence of a flagella. However, lower levels of post-thaw viability from both the intermediate

temperature (-60°C) and LN for *E. mutabilis* and *A. longa* may be because protocols require further adaptations in order to optimise them to yield maximum post-thaw viability levels for these organisms (Table 3.9).

The protocol developed for *E. gracilis* has been applied with equal success for the preservation of *E. gracilis* at the Institute of Freshwater Ecology and at The University of Abertay Dundee, using programmable freezers which differed in age, model and style. This indicates that the optimum protocol, which employed a cryoprotectant solution of methanol at 10% (v/v) added at 0°C, with a 15 min. incubation period, prior to cooling at -0.5°C $min.^{-1}$ to -60°C with a 30 min. holding step prior to plunging directly into LN and a two-step thawing protocol, was robust.

3.4.4 *Vaucheria sessilis*

It was possible to expose filaments of *V. sessilis* to a number of cryoprotectant solutions at concentrations of 5 an 10% (v/v) without any loss in viability (Table 3.7). Methanol at 10% (v/v) and DMSO at 5% (v/v) were selected because of their previous successful application, both in this study and other published cryopreservation procedures (Meryman, 1971).

The ability of *V. sessilis* to retain full metabolic capability and grow after being exposed to 0°C and supercooled media (-10°C) demonstrated that *V. sessilis* was not simply chill sensitive (Fig. 3.9, Table 3.8). The findings of this study suggest that an optimal cooling rate may exist for *V. sessilis*. Furthermore, detailed investigations into chilling and freezing injury/damage in *V. sessilis* are detailed in Chapters 4 & 5.

3.4.5 Long-term maintenance of algal cultures

During this study no reduction in viability was detected over 12 months of cryostorage in the alga *H. pluvialis* (3.3.3). This was consistent with the observation that in members of the algal classes: Chlorophyceae, Prasinophyceae, and Xanthophyceae no significant changes in viability detected, for storage periods of up to 22 years (Day *et al.*, 1997). The results demonstrate that cryopreservation has the potential to be employed for the

long-term storage of genetic resources. Stable storage, for prolonged periods of time has significant implications for the preservation of biological materials including human sperm and embryos and in the preservation of patent deposits of living material under the Budapest Treaty (Anonymous, 1995).

There was no evidence of any significant changes in temperature during storage (Fig. 3.13). Assuming there were no deviations from the routine addition of liquid nitrogen, the thermal behaviour of the cryostore should have be very stable over time. However, cells for cryopreservation require to be maintained for extended periods. It is therefore possible that cell damage may occur on manipulation of the cryovial. Temperatures of up to -112°C were observed on removal of cryovials from the bottom of the inventory system (Fig. 3.14). The duration of the exposure of vials to the elevated temperature was extremely brief. However, at this temperature damage could theoretically occur to stored material and the possibility of devitrification and recrystallisation are discussed more fully in 1.9.2. Although recrystallisation generally occurs at high subzero temperatures, it has previously been detected as low as -130°C and as low as -150°C with the aid of X-ray diffraction techniques (Taylor, 1987; MacFarlane *et al.,* 1992). Free-radical mediated damage has also been implicated in freeze-induced damage in both plant and animal systems (Benson, 1990). Although most free-radical mediated damage is likely to occur at warmer temperatures, free-radical activity has been demonstrated in systems cooled to -80°C (Whiteley *et al.*, 1992a,b). In this study temperatures as high as -80°C were not encountered during addition/retrieval manipulations. However, there is a risk of these higher temperatures being reached if the frozen material is manipulated using suboptimal or slower procedures.

3.4.6 Conclusions

Two-step cryopreservation protocols developed using programmable cooling technology offer the possibility of developing robust cryopreservation protocols for the algae. However, each algal strain is likely to require the development of a specific cryopreservation protocol in order to allow the highest levels of post-thaw viability to be achieved. The stability of cryopreserved material has been previously reported by Day *et al.* (1997) and has been confirmed for the preservation of *H. pluvialis* over a 12 month

period. The observed stability of the temperature regime within the cryostorage facilities at CCAP further promotes the use of cryopreservation for the long-term maintenance of algal cultures. However, temperature fluctuations detected during vial manipulation studies are potentially high enough to present the possibility that damage may occur in preserved material. In working collections, it would therefor be prudent to maintain a separate back-up culture bank which did not undergo regular routine manipulation. Presently, *E. gracilis*, *H. pluvialis*, *E. intestinalis* and *M. aeruginosa* may be recovered after exposure to LN with viability levels > 40% of untreated controls.

Cryoinjury has been attributed to a series of complex events and may be affected by the cooling rate employed, the period of exposure to cryoprotectant and elevated solute concentrations. It is, however, important not to discount the importance of other cryoinjurious factors (*e.g.*, oxidative stress and biochemical events) these events are investigated and discussed in more detail in subsequent chapters.

Chapter 4.

Visual investigation of cellular damage and recovery.

Contents **Page No.**

4.1 Introduction

To further current understanding of cryobiology, improve levels of post-thaw viability and cryopreserve presently freeze-recalcitrant organisms, there is a requirement to adopt more investigative techniques in preference to the empirical approach which has largely been employed to date. More investigative procedures would allow points of cryoinjury, or stress, to be identified and better understood. This would then permit the development of cryopreservation protocols by "designing out" these injurious events and stresses. The results discussed in Chapter 3 were largely derived via empirical investigations and although a number of hypotheses were developed regarding lethal injury in the strains studied no direct investigations were carried out to quantify these cryoinjurious events.

Visualisation of the effects of chilling and freezing on algae is an approach which permits the investigation of mechanisms of cryoinjury and their effect on post-thaw recovery. Investigation of visible changes which occur in cells during a freezing protocol may highlight and allow specific points of cellular injury to be identified. Through visual exploration of cryopreserved material it is thus possible to direct further research towards possible sites of cryodamage, *e.g.*, at the biochemical level, thus potentially permitting the development of better cryopreservation protocols (Morris *et al.*, 1988a,b).

Since the development of early light microscopes by Antoni van Leeuwenoek, permitted the observation of living protozoa in the late 17^{th} century (Dobell, 1960), there has been the possibility of applying microscopical techniques to the investigation of cryoinjury at the cellular level. Even these early light microscopes were capable of observing structures as small as red blood corpuscles and protozoa, in addition, they were capable of resolving fibrillar structures of less than 1μm thick (Van Zuylen, 1980). The application of conventional light microscopy to the investigation of cryoinjury, allows comparisons to be made between cells pre- and post-treatment. In addition, through the implementation of vital and mortal stains, post-treatment cellular viability and injury may be detected. Microscopy allows non-lethal changes and gross injury to be visualised

including: membrane rupture, displacement of the cell wall and common stress responses in cells, *e.g.*, morphological changes and/or flagellar loss. Furthermore, cells examined post-thaw, may have gaps in their cytoplasm which may be due intracellular ice, or gas bubbles, which had formed in the intracellular space during the cryopreservation protocol. Furthermore, flagellate organisms which rapidly recover from cryopreservation, after a short lag phase, may be observed to regain motility.

Cryomicroscopy, a technique developed from light microscopy allows the researcher to visualise real time events as they occur during the cryopreservation protocol. As early as 1897, cryomicroscopy enabled Molisch, to conclude that cellular death due to freezing could be attributed to dehydration caused by the formation of extracellular ice (McGrath, 1987). These observations together with qualitative studies of severe dehydration in *Spirogyra* spp. as a result of extracellular freezing, intracellular ice formation in *Amoeba* spp. and the recognition that there was an important link between solute concentration and cell death were all performed using a simple low-temperature microscope (McGrath, 1987). With the advent of electronics the possibility of regulating the temperature on a microscope stage using cryogenic fluids (LN or LN vapour) became possible and the modern cryomicroscope was developed.

During the course of this research two styles of cryomicroscope were employed. The first cryostage was the Planer CM3 Cryostage (Planer, UK) which was cooled by LN vapour driven by head pressure in the supply Dewar via a heat sink (a copper coil immersed in LN). To observe samples the cells were first mounted then placed on a transparent heater in the centre of the stage. The rate of cooling was regulated by the thermal gradient which existed across the cryostage and was determined by the difference between the thermocouple at the stage centre and the surrounding cooling block (Fleck *et al.*, 1997a). Cooling was controlled by programming a series of "ramps" which regulated: rates of temperature change, the temperature limit for the cooling rate and the holding period at the temperature limit. The second cryostage was a Linkam BCS 196 (Linkam, UK) unit which was cooled by drawing LN from a 2 l LN Dewar. By employing this arrangement it was possible to avoid the formation of thermal gradients across the stage permitting supercooling of the media. As in the Planer CM3 unit cooling was electronically controlled.

Cryomicroscopy permitted the monitoring of real-time intra- and extracellular events associated with chilling and freezing. Furthermore, electronic regulation allowed the controlled manipulation of the temperature on the stage of a light microscope, there by allowing the duplication of the conditions experienced during two-step cryopreservation protocols. Using the information generated, freezing protocols may then be developed and adapted to minimise cell damage and thus increase post-thaw viability (Morris *et al.*, 1988a,b).

Transmission electron microscopy (TEM) permited the visualisation of intracellular events post-treatment. The sectioning of fixed material allowed ultrastructural changes and changes in chemical inclusions to be visualised. However, the preparation of biological tissue for transmission microscopy requires that the tissue first be stabilised to enable it to survive the severe environment within the transmission electron microscope, *e.g.*, being maintained in a high vacuum and being bombarded with highly accelerated electrons. Traditional TEM methods involve the fixation of the tissue with chemicals, *e.g.*, aldehydes which stabilise the components of the cell by molecular cross-linking, followed by the dehydration of the tissue by the substitution of the aqueous proportion of the tissue with non-aqueous solvents, *e.g.*, ethanol. The tissues are then embedded in a suitable resin before being sectioned using an ultramicrotome. Staining of the sections can be performed to increase image contrast and make the identification of organelles easier.

4.1.1 Objectives

By carrying out a series of cryomicroscopic investigations on the key organisms used in this study and employing optimum and suboptimum freezing regimes it was anticipated that a greater understanding of freezing stresses and injury could be obtained. It was also hoped that this would lead to the possibility of developing more robust freezing protocols. In addition, a further objective was to employ electron microscopy to investigate ultrastructural changes and damage in *E. gracilis* and *V. sessilis*.

4.2 Materials and methods

4.2.1 Organisms and culture regimes

The strains selected for study (*Haematococcus pluvialis* Flotow CCAP 34/8, *Enteromorpha intestinalis* (L.) Link CCAP 320/1, *Euglena gracilis* Klebs CCAP 1224/5Z and *Vaucheria sessilis* (Vaucher) De Candolle ex Collins CCAP 745/1C) are detailed in 2.1. Culture regimes and recovery conditions were as described in 2.2-2.3.

Filaments of the xanthophytic alga *Vaucheria sessilis* were sectioned into 12 mm lengths, then incubated under standard conditions for 48 h., prior to cryopreservation/cryomicroscopy (Fleck *et al.*, 1997a).

4.2.2 Cryopreservation procedures

Cryoprotectant solutions were always added to cell suspensions, to avoid excessive toxic shock. Cryoprotectant chemicals employed were: dimethylsulphoxide (DMSO) and methanol (Sigma, USA). Final concentrations of 5 or 10% (v/v) of cryoprotectant were used throughout, with a 5 or 15min. exposure period at room temperature (RT/20°C) or at 0°C prior to cooling to subzero temperatures.

Vials containing 0.5ml of algae/cryoprotectant were frozen employing controlled cooling methodologies (2.4). Vials containing cells for controlled cooling were cooled to their intermediate holding temperature using a Planer Kryo 10 programmable freezer (Planer, UK). Cooling rates of -1°C $min.^{-1}$, -0.5°C $min.^{-1}$, and -0.3°C $min.^{-1}$ were employed. Vials were cooled to -35°C or -60°C and held at their intermediate temperature for either 15 and 30 min. periods, prior to being plunged directly into LN.

Vial contents were thawed using a single step direct immersion in a pre-heated water bath at 40°C, or with a slow warming step of 1 min. in the air (room temperature) followed by rapid warming in a pre-heated 40°C water bath. All vials were agitated until the last ice crystals had melted (Day *et al.*, 1997). The thawed material was then aseptically transferred to appropriate fresh, sterile, medium (2.2), and used in the

viability assays as outlined in 2.5. All data are the mean of three assays and errors are expressed as standard errors of mean.

4.2.3 Light microscopy

Light microscopy was carried out as described in 2.6.1 using vital and mortal stains as described in 2.5.2.

4.2.4 Cryomicroscopy

Sectioned filaments or cell suspensions were examined using a Linkam BCS 196 Cryostage mounted on either an Olympus BX-50 microscope or a Leitz Dialux 22 microscope with an adapter unit modified from a standard Leitz stage (Fleck *et al.*, 1997a). Alternatively a Planer CM3 cryostage mounted on an Olympus BH-22 microscope (2.6.2-2.6.2.2) was employed. Video and still images were collected as described in 2.6.2.

4.2.5 Volume analysis

Measurements of changes in filament width and area were carried out with the aid of the Linkam video text overlay unit VTO 232. The VTO was calibrated for the lenses attached to the Olympus BX-50 and Leitz Dialux 22 microscopes as detailed in the Linkam VTO 232 Reference manual (Linkam Scientific Ltd., UK). The calibrated unit was then used in area measurement and line measurement modes and the resulting data expressed in μm or μm^2 (Fleck *et al.*, 1997a).

4.2.6 Transmission electron microscopy

TEM studies were performed on fixed material embedded in Spurr resin as described in 2.6.3 & 2.6.3.2. In all cases material was fixed immediately post-treatment.

4.2.6.1 TEM of *Euglena gracilis*

Algal cells were fixed at room temperature for 60 min. in (0.1M sodium cacodylate buffer) buffered glutaraldehyde [4% (w/v)]. Cells then underwent two buffer washes, followed by a 30 min. fix in buffered osmium tetroxide [2% (w/v)], followed by a further buffer wash. Cells were centrifuged and supernatant removed between each fixation step.

Fixed filaments were dehydrated in a graded ethanol series to 100% ethanol and in 100% ethanol. Material was then embedded for ultramicrotomy. Embedding was carried out with a two step graded increase in Spurr resin from 50% (v/v) to 100% Spurr. Samples were incubated overnight at room temperature, at each series step and finally cured at 60°C. Sections of the embedded material were cut on a ultramicrotome, and examined as described in 2.6.3.2.

4.2.6.2 TEM of *Vaucheria sessilis*

Algal filaments were fixed at room temperature for 15 min. in buffered (0.1M sodium cacodylate buffer) paraformaldehyde [2% (w/v)], followed by a further 60 min. fix in buffered glutaraldehyde [2% (w/v)]. Filaments then underwent two buffer washes, followed by a 60 min. fix in buffered osmium tetroxide [2% (w/v)], followed by two further buffer washes, two deionised water washes and a final 60 min. dark fix in aqueous uranyl acetate [2% (w/v)].

Fixed filaments were dehydrated in a graded ethanol series to 100% ethanol and in 100% ethanol. Material was then embedded for ultramicrotomy. Embedding was carried out with a three step graded increase in Spurr resin from 25% (v/v) to 100% Spurr. Samples were incubated overnight at room temperature, at each series step and finally cured at 60°C. Sections of the embedded material were cut on a ultramicrotome, and examined as described in 2.6.3.2.

4.2.7 Scanning electron microscopy

4.2.7.1 SEM of *Euglena gracilis*

Preparation of *E. gracilis* for scanning electron microscopy (SEM) followed a specific fixing protocol to avoid loss of flagella. A Buchner funnel was prepared to allow a cell suspension to be supported above a 0.2μm Nuclepore filter. *E. gracilis* suspension (2 ml) was then added to the filter unit. Cells were fixed by the rapid addition of buffered paraformaldehyde (5 min.) followed by the addition buffered gluteraldehyde (15 min.) to final concentrations of 2% (v/v). Excess liquid was then drawn off using a vacuum pump while being continuously diluted with sodium cacodylate buffer (5 ml). This procedure was repeated three times and material was then further fixed with osmium tetroxide (2% v/v) for 15 min. Fixed cells were then subjected to 5 water washes and then dehydrated in alcohol. Cells were maintained in suspension at all times preventing flagellum loss, fixatives and alcohol steps were as described in 2.6.3. SEM was performed on critical point dried *E. gracilis* cells coated with gold as described in 2.6.3.1.

4.3 Results

4.3.1 *Euglena gracilis*

Euglena gracilis is a flagellate euglenoid, with distinctive pellicle structure and cells are normally elongate in form (Fig. 4.1).

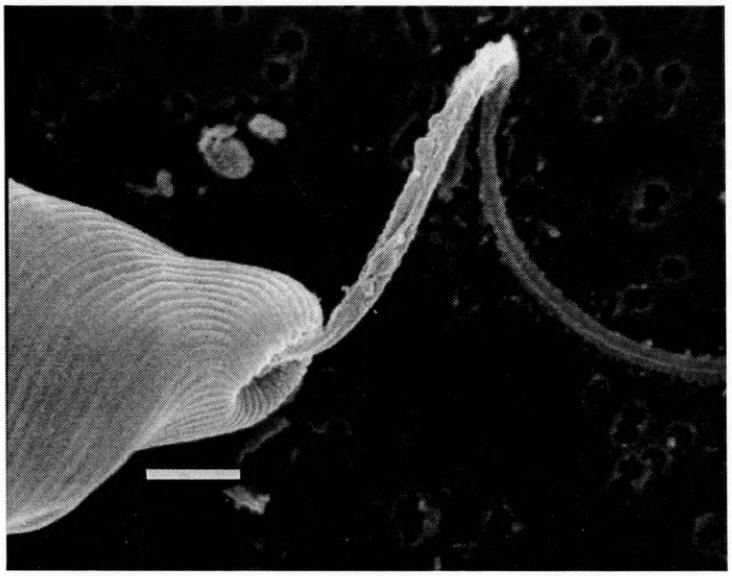


Figure 4.1 SEM micrograph of *Euglena gracilis* showing flagellum, flagellar insertion point and the ribbed pelicle structure of the cell.

Scale bar represents 1μm.

Cryomicroscopic studies have demonstrated that the cells will lose their elongate form and become spherical (a common stress response) after prolonged exposure to cryoprotectant solutions (10% (v/v) methanol) and as the temperature drops to 0°C (Fig. 4.2). Microscopic studies on thawed material commonly allowed observation of non-flagellate cells with separate flagella complete with intact basal bodies. No intact cells with flagella were observed during the initial stages of post-thaw recovery. During cooling, cells were observed to undergo a series of responses promoted by exposure to the frozen environment.

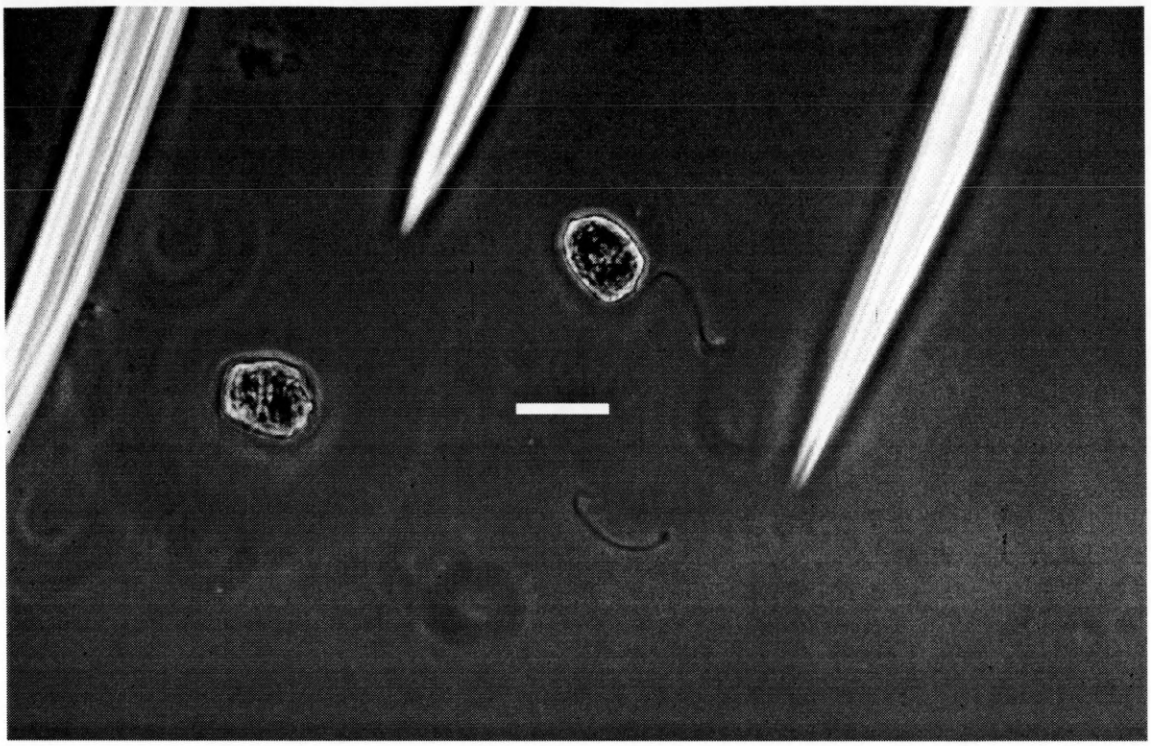


Figure 4.2 Cryomicroscopy image of *Euglena gracilis* cells at -1°C. One cell has already lost its flagellum.

Scale bar represents 60μm.

In the extracellularly frozen system, cryodehydration of the cells was observed and could be quantified by using pixel counting techniques (Figs. 4.3, 4.4). Cells were commonly dehydrated to 72% ± 5.0% of their original volume during cooling at -0.5°C min.$^{-1}$ to the intermediate holding point (-60°C) (Fig. 4.4). Furthermore, where rapid cooing rates were employed (-10°C min.$^{-1}$), and lethal intracellular ice nucleation events were observed during cooling to the intermediate holding temperature (-60°C), the volume of the cells post-thaw expanded to >100% of their original volume [Fig. 4.3 (Rate 3)].

For *E. gracilis* the rate of cooling to the intermediate holding temperature (-60°C) influenced the temperature of intracellular ice nucleation (Table 4.1). Rapid cooling at rates of >-1°C min.$^{-1}$ resulted in intracellular ice nucleation in 100% of the cells observed. The more rapid the cooling rate, the higher the subzero temperature at which intracellular ice nucleation was detected (Table 4.1). In cells cooled at -0.5°C min.$^{-1}$ a small proportion (5%) of cells were observed to experience intracellular ice nucleation.

However, on employing a slower cooling rate (-0.3°C min.$^{-1}$) no cells were observed to experience intracellular ice nucleation (Table 4.1).

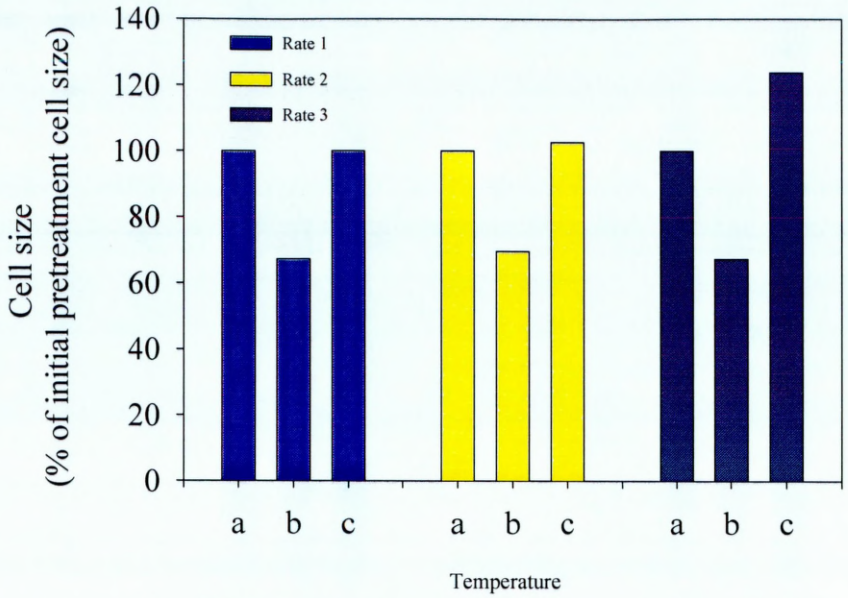


Figure 4.3 Change in cell size during two-step controlled cooling of *Euglena gracilis* at different controlled cooling rates.

Rate 1 = -0.5°C min.$^{-1}$ Rate 2 = -0.3°C min.$^{-1}$ Rate 3 = -10°C min.$^{-1}$ (a) 0°C, (b) -60°C (c) 25°C.

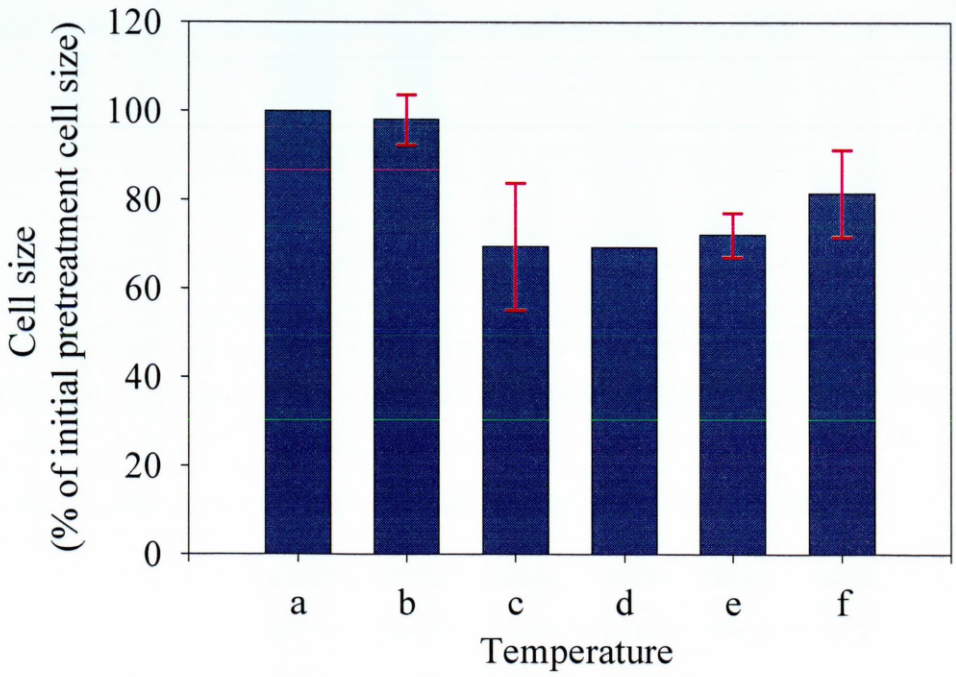


Figure 4.4 Change in cell size of *Euglena gracilis* during optimal two-step cooling protocol during controlled cooling to -60°C.

(a) 0°C, (b) -10°C, (c) -28°C, (d) -55°C, (e) -60°C, (f) 25°C.

n = 6, errors are expressed as standard errors of mean.

Table 4.1 Effect of cooling rate on *Euglena gracilis*

Cooling rate [a]	Intracellular ice [b]	% [c]	Temp (°C) [d]	Fracture [e]
-20	+	100	-15	+
-10	+	100	-26	+
-5	+	100	-28	+
-1	+	100	-30/-100 [f]	+
-0.5	+	5	-37	+
-0.3	-	0	NA	+

[a] Controlled cooling rate to -60°C (°C min.$^{-1}$), and at -100°C min.$^{-1}$ to -170°C.
[b] Intracellular ice observed
[c] % of cells containing intracellular ice
[d] Temperature of intracellular ice nucleation event
[e] Freeze fracture event observed
[f] Some cells underwent intracellular ice nucleation at -35°C with the remaining cells undergoing intracellular ice nucleation at -100°C
+ Event observed
- No event observed
n = 3

Ultrastructural changes in *E. gracilis*. Untreated, control cells contained many regularly arranged chloroplasts. The chloroplasts had an elongate form and contained pyrenoids and parallel-orientated thylakoids, without grana organisation. The inter-thylakoid spaces were narrow (Fig. 4.5). Mitochondria often appeared to be associated with chloroplasts and were dense in aspect with cristae occupying their entire volume (Fig. 4.5) and nuclei were uniform and dense in aspect (Fig. 4.5). A number of additional structures were observed including: endoplasmic reticulum and starch and lipid inclusions (Fig. 4.5).

Cooling and freezing to -60°C resulted in no detectable changes in organelle form or distribution and no increase in lipid deposits were observed in frozen material [Fig. 4.6 (A, B)]. Chloroplasts appeared normal and were indistinguishable from those in control cells.

After a freeze-thaw cycle to LN the cellular structure appeared normal. Mitrochondria, chloroplasts lipid inclusions and the nucleus were all readily identifiable [Fig. 4.6 (A)]. In cell sections thawed after exposure to -60°C and LN temperatures there was increased vesiculation and evidence of disruption of the cytosol. This was commonly associated with starch and lipid inclusions [Fig. 4.6 (A)]. Within these cells the chloroplasts thylakoid arrangement did not appear noticeably modified, in addition, the mitochondria and their cristae also appeared normal [Fig. 4.6 (A)]. In cells which had been exposed to a direct plunge into LN (-196°C), the cells had similar, but exaggerated, damage when compared with cells which had been frozen using a two-step protocol. Cells commonly had a high degree of vesiculation, in addition, the nucleus and the mitochondria appeared less dense with the loss of cristae structure, furthermore, chloroplasts appeared modified and had lost much of their thylakoid structure [Fig. 4.6 (B)].

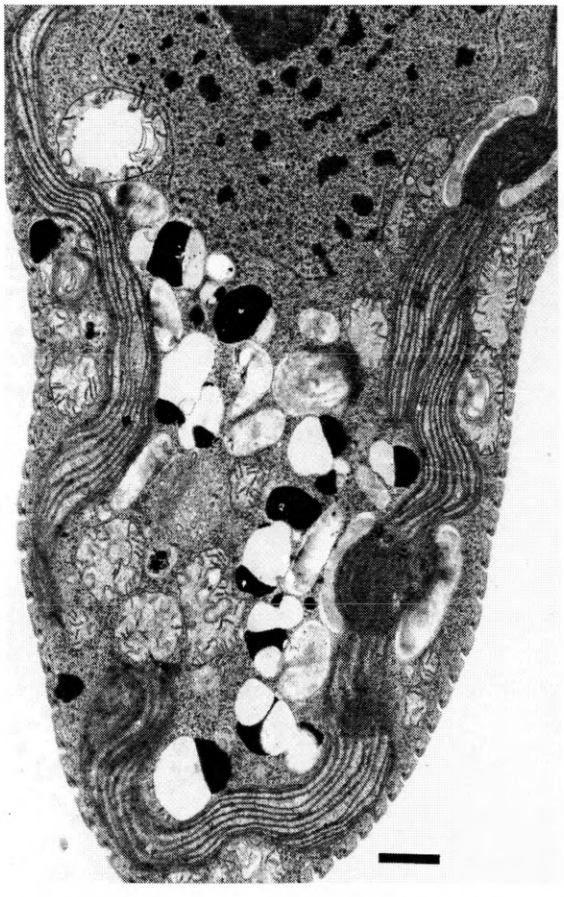
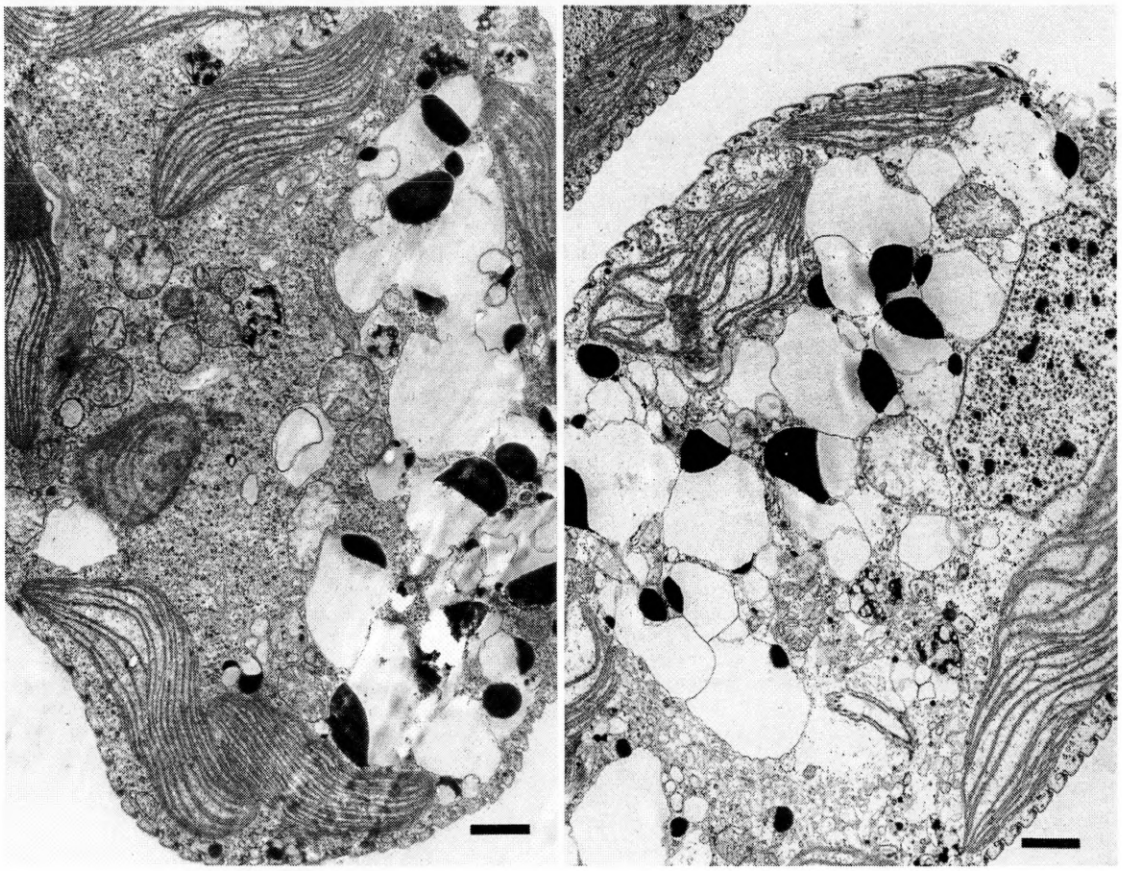


Figure 4.5 TEM micrographs of *Euglena gracilis* untreated control.

Scale bar represent 1μm.



A B

Figure 4.6 TEM micrographs of *Euglena gracilis* (A) Cooled at -0.5°C min.$^{-1}$ to -60°C in the presence of cryoprotectant [10% methanol (v/v)], (B) Plunged directly into LN without cryoprotectant.

Scale bars represent 1μm.

4.2 *Haematococcus pluvialis*

Cryomicroscopic studies at the optimum cooling rate of -1°C min.$^{-1}$ to -35°C permitted visualisation of the effect of cryodehydration on the aplanospore stage *H. pluvialis* cells. The cells had a distinct brick red centre containing a high concentration of lipid soluble carotenoid [Fig. 4.7 (A)]. The cell membranes of the aplanospore were surrounded by thick, relatively impermeable cell walls [Fig. 4.7 (A-D)]. During controlled cooling the volume of the red centre was reduced to >40% of its original volume [Fig. 4.7 (A-C)]. On thawing the contracted cell interior could clearly be seen within the non-contracted cell wall. Immediately post-thaw the intracellular compartment rehydrated to >80% of its original volume. This could also be visualised as a decrease in the distance between the outer cell wall and the inner cell membrane. The distance between the cell wall and

the cell membrane increased by >250% through cryodehydration, whilst in the frozen environment, and was reduced within 2 min. of thawing to <150% of the distance measured prior to cooling [Fig. 4.7 (A-D)].

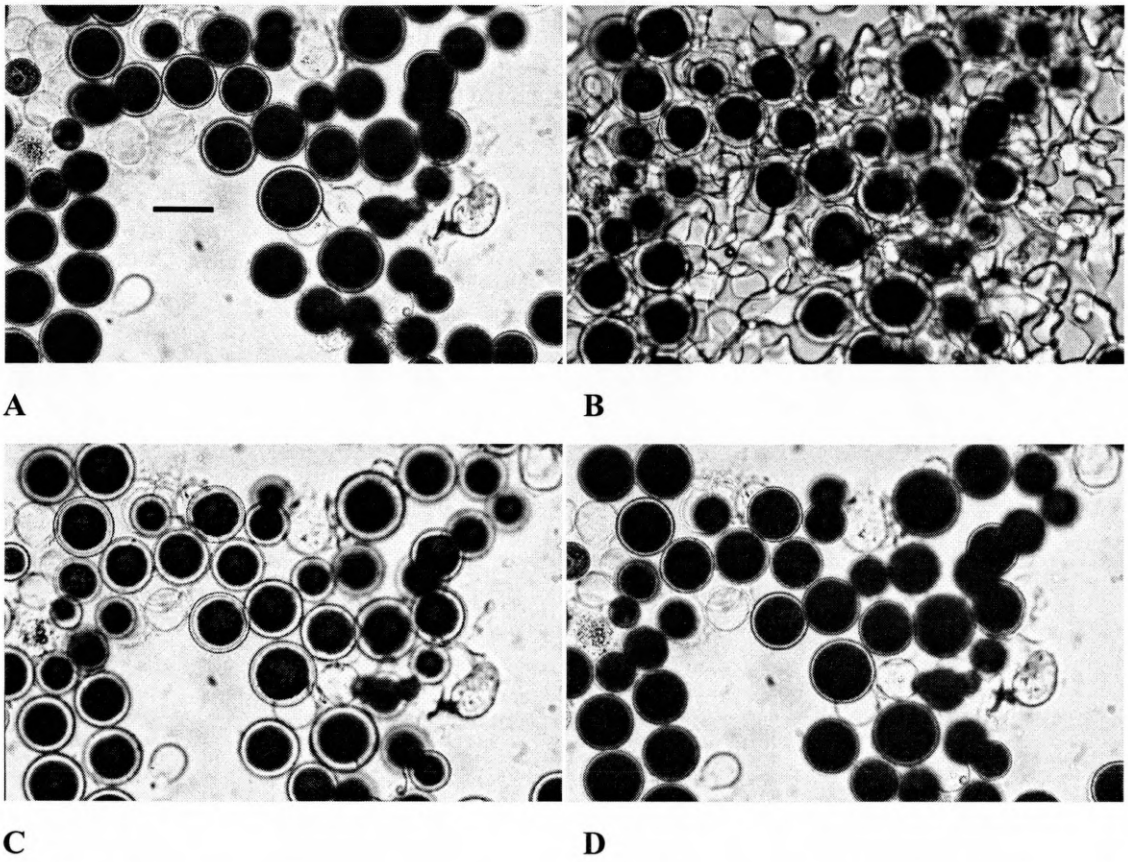


Figure 4.7 *Haematococcus pluvialis* (A) 20°C, (B) During melt, (C) 25°C immediately post-thaw, (D) 10 min. post-thaw.

Scale bar represents 80μm.

By employing suboptimum, rapid cooling rates (-10°C min.$^{-1}$ & -20°C min.$^{-1}$), intracellular ice nucleation could be induced in cells during cooling to the intermediate holding temperature (-35°C) (Table 4.2). The most rapid cooling rate -20°C min.$^{-1}$ resulted in a higher proportion of cells experiencing intracellular ice nucleation on cooling to -35°C (80%), compared with 25% and 0% in cells cooled at -10°C min.$^{-1}$ and -1°C min.$^{-1}$ respectively (Table 4.2).

Table 4.2 Effect of cooling rate on *Haematococcus pluvialis*

Cooling rate [a]	Intracellular ice [b]	% [c]	Temp (°C) [d]	Fracture [e]
-20	+	80	-35	+
-10	+	25	-35	+
-1	+	10	-120	+

[a] Controlled cooling rate to -35°C (°C min.$^{-1}$), and at -100°C min.$^{-1}$ to -170°C.
[b] Intracellular ice observed
[c] % of cells displaying intracellular ice
[d] Temperature of intracellular ice nucleation event
[e] Freeze fracture event observed
+ Event observed
- No event observed
n = 3

During repeated freezing runs, intracellular ice nucleation events were not always observed in cells cooled at -1°C min.$^{-1}$ to -35°C then at -100°C min.$^{-1}$ to -170°C, was attributed to the difficulty in achieving a uniform rapid secondary cooling rate of -100°C min.$^{-1}$.

4.3.3 *Vaucheria sessilis*

After exposure to cryoprotectant solutions at 0°C and 22°C filaments of *V. sessilis* demonstrated normal cytoplasmic streaming and regrowth. In addition, *V. sessilis* was able to recover from the effects of the cooling regime; cryoprotectant toxicity, dehydration and partial plasmolysis down to -10°C, in the absence of extracellular ice, (Table 3.8). On thawing frozen material, no recovery of metabolic capability or filament growth was observed (Table 4.3). No improved tolerance to the effects of cryopreservation was induced by pre-culture on solid media, or through the use of solid or liquid pre-culture media supplemented with 0.09M Sucrose or 1.2M Sorbitol. Furthermore, on prolonged incubation under a standard culture regime the filaments

bleached due to the degradation of chlorophyll and other photosynthetic pigments (Table 3.8).

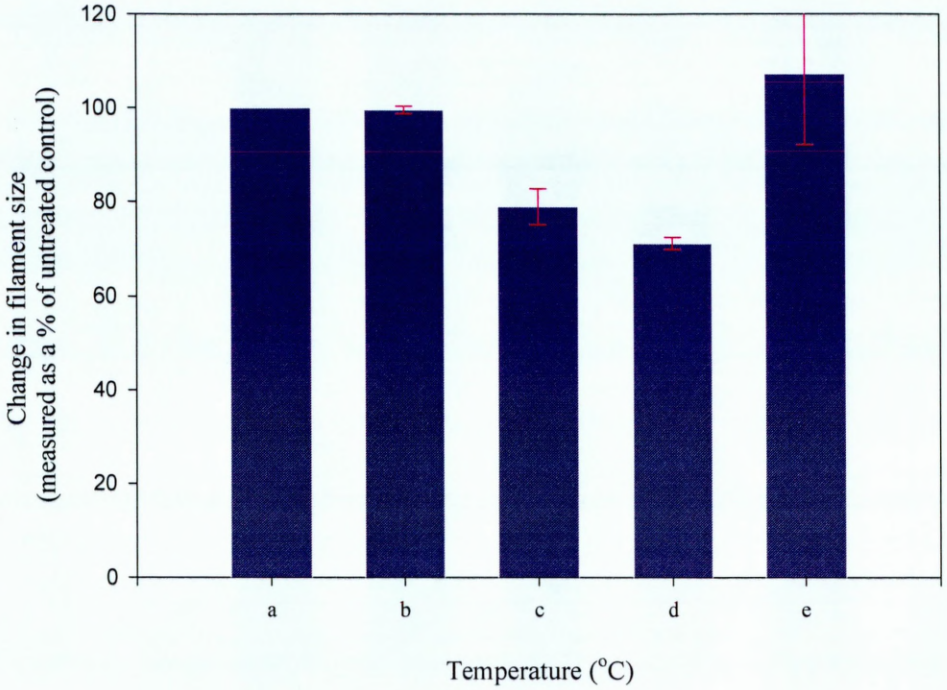


Figure 4.8 Change in filament volume in *Vaucheria sessilis* during a two-step cryopreservation protocol.

Filaments pre-treated with cryoprotectant (10% (v/v) methanol) and cooled from 0°C to -35°C at -1°C min.$^{-1}$, then at -100°C min.$^{-1}$ to -150°C. Filaments were then warmed at 80°C min.$^{-1}$ to 25°C. (a) 0°C, (b) -16°C, (c) -24°C, (d) 22°C, (e) 25°C.

Results are expressed as the mean of 5 observations with standard errors of mean.

Mechanisms of lethal freeze-induced injury were investigated using cryomicroscopy. At a cooling rate of -1°C min.$^{-1}$ significant dehydration of the filament occurred before intracellular freezing [Figs. 4.8, 4.9 (B, C)]. Using the Planer CM3 cryostage, ice nucleation in the sample occurred between 0 and -3°C, with further cryodehydration of the cell down to the holding temperature [Fig. 4.8, 4.9 (C)]. In addition, the filaments were crushed by extracellular ice, resulting in distortion of the cell wall [Fig. 4.9 (C)]. Intracellular ice nucleation was not observed in the cytosol using the Planer cryostage in specimens cooled to -35°C or -60°C and no intra- or extracellular gas bubbles were noted on cooling or warming. On thawing, all specimens showed signs of mechanical damage and distortion of their intracellular architecture [Fig. 4.9 (D)].

From studies of the exothermic events encountered during the controlled cooling of material in a Planer Kryo 10, it was established that ice-nucleation of the extracellular solution, (as identified by a thermocouple inserted in a vial containing culture medium and cryoprotectant) occurred at -13°C ± 0.9°C and -14°C ± 1.5°C with the cryoprotectants DMSO and methanol respectively (3.3.2). This clearly indicated the medium underwent a considerable degree of supercooling during this first controlled cooling stage of a two-step cooling protocol. To duplicate these supercooling events the alga was investigated using a Linkam BCS 196 cryostage, without seeding. There was a visible degree of dehydration of the filaments prior to freezing. However, as no mechanical ice-seeding was initiated, supercooling occurred, with the medium freezing at between -15 and -19°C (Table 4.4). In addition, on using this system, intracellular ice was observed, as evidenced by "flashing" in the majority of filaments. With the aid of video imaging it was possible to observe the "flashing" events in detail. Thus, in many cases, following nucleation at one location within the filament an ice front could be seen to travel rapidly along the length of the filament, the associated darkening of the intracellular space was considered to indicate growth of the ice-front (Table 4.4).

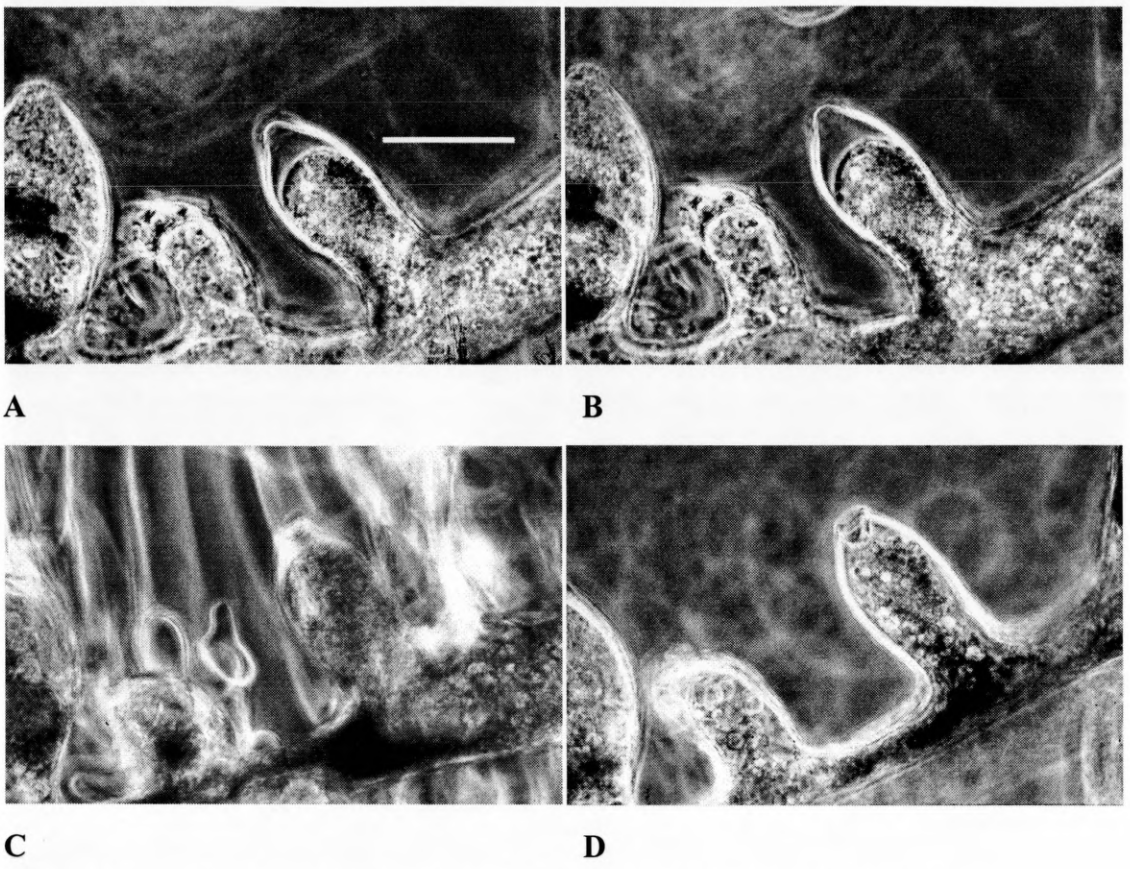


Figure 4.9 *Vaucheria sessilis* (A) 25°C, (B) - 8°C, (C) -6°C, (D) post-thaw.

Scale bar represents 100μm.

Using video-based measurements, cryodehydration and ice crushing of the filaments were observed, which could account for as much as a 50% reduction in the filament volume. The majority of this reduction occurred between the point of extracellular ice nucleation and the intermediate subzero holding point (-35 or -60°C), with little further change in volume below this point. The effects of cryodehydration and filament crushing were much greater than the small degree of dehydration occurring prior to freezing of the extracellular solution. Filaments rapidly expanded/rehydrated on thawing from their frozen state with many filaments rehydrating to greater than 100% of their original volume suggesting the ultrastructure of the cell wall had been damaged (Fig. 4.8).

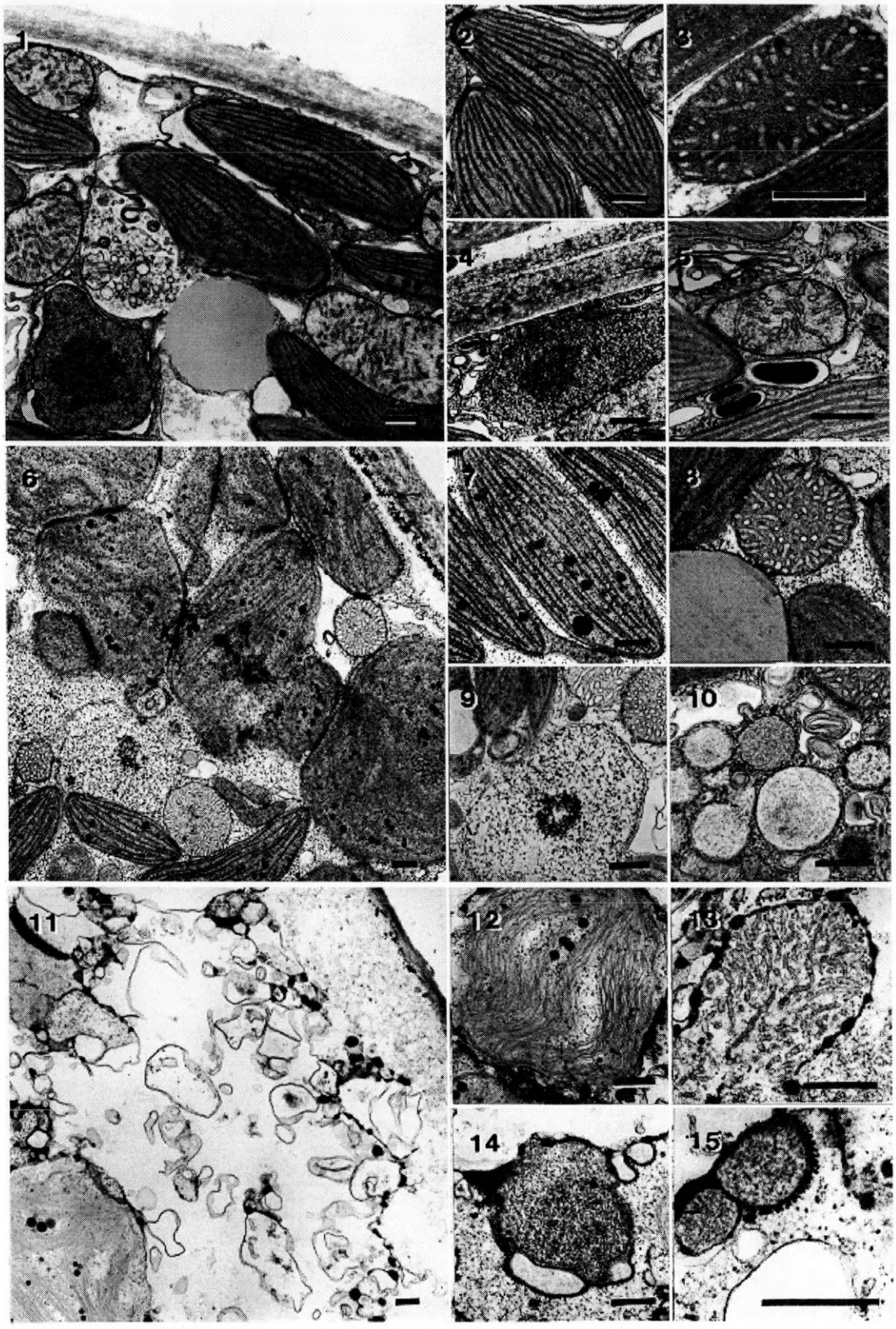


Figure 4.10 TEM micrographs of *Vaucheria sessilis*; (1-5) Control, (6-10) -10 °C, (11-15) -60 °C: showing distribution of cellular organelles and filament wall structure. (2,7,12) Chloroplast. (3,8, 13) Mitochondria. (4,9) Nucleus (5,10) Microbodies (14) vesiculated intracellular material/microbody, (15) Vesiculated rough endoplasmic reticulum.
Scale bars represent 0.5μm.

Cooling *V. sessilis* to subzero temperatures induced different responses in the alga dependant upon cooling rate employed and the temperature to which the specimen was cooled.

Metabolic effects of each step in cryopreservation protocols employed were investigated in *V. sessilis* by looking at their impact on the filaments photosynthetic capacity. Exposure to the cryoprotectants reduced the photosynthetic capacity of *V. sessilis*, by 70 ± 10%, when they were added at 0°C, and by 90 ± 3%, when added at room temperature (Fig. 3.9). However, filaments subsequently recovered fully on removal from the cryoprotectant solution (Table 4.3). Where filaments were cooled to subzero temperatures, and the extracellular environment froze, an initial depression of the photosynthetic capacity by > 80% occurred (Fig. 3.9). On further incubation under standard culture conditions oxygen evolution rates returned to close to 100% (Table 4.3).

Cooling the filaments until intracellular ice occurred, resulted in total loss of oxygen evolution, these filaments ultimately bleached and died. In all cases where filaments retained the capacity to evolve oxygen, they were able to grow and could generate fresh filaments (Table 4.3). No significant bleaching, or variation in chlorophyll concentration, was noted during the recovery of these filaments; [Time zero 680 ± 50 μg Chl. *a*. g^{-1} (wet weight). 24 h. post-treatment; 680 ± 60 μg Chl. *a*. g^{-1} (wet weight). 48 h. post-treatment; 630 ± 30 μg Chl. *a*. g^{-1} (wet weight)].

Visualisation of the effects of cooling in *V. sessilis* employing cryomicroscopy allowed changes in cooled material to be identified. These were then categorised as either independent or dependant on cooling rate [Fig. 4.9 (A-D), Table 4.3, 4.4].

Changes independent of cooling rate included:

The sample (medium and algal filament) underwent initial supercooling, with the external medium and intracellular aqueous environment cooling to subzero temperatures without an ice nucleation event (Table 4.3). No measurable change in filament volume

was detected prior to extracellular ice nucleation. Immediately after extracellular ice nucleation (-12°C → -18°C), the cell volume was reduced to 60 ± 11% of its original volume. Further cryodehydration of the filament was observed down to the point of intracellular ice nucleation, by which time filaments were reduced to 50 ± 2% of their original volume.

Freezing of the external solution caused a number of additional responses including; localised darkening of the cytoplasm due to compression and dehydration of the filament (Table 4.3) and rapid displacement of organelles within the filament due to extracellular ice crushing (Table 4.3). Intracellular freezing was also clearly observable in all filaments and commonly took the form of an ice front travelling the length of the filament.

Changes dependant upon cooling rate and cryomicroscopic observations included:

The cooling rate employed affected the temperature at which intracellular ice formation was observed (Table 4.4). More rapid cooling rates gave rise to readily observable intracellular ice formation at higher subzero temperatures. On cooling at -1°C $min.^{-1}$, narrowing of the filament in the frozen environment was observed. This was particularly apparent between -15 and -17°C. Movement of intracellular organelles was observed between -17.4 and -18.4°C, with localised darkening of the cytoplasm. Further darkening was observed between -35.0 and -70.0°C with a distinct ice front running the length of filament at -80°C. This was described as a flashing event (Table 4.4).

Cooling at -10°C $min.^{-1}$ resulted in darkening of the filament due to the concentration of intracellular material by external ice crushing and/ or by cryodehydration. This was observed between -21 and -22°C. Further darkening of the filament, caused by intracellular ice "flashing", was observed between -21.9 and -30.0°C (Table 4.4).

On cooling at -20°C $min.^{-1}$, crushing of the filament by extracellular ice, cryodehydration, or localised intracellular ice formation was observed to caused the displacement of intracellular organelles. Rapid movement of cytoplasm and organelles

with a distinctive "mixing" action was observed between -20 and -26°C. Localised darkening was also noted between -21.2 and -26.2°C. An ice front running the length of the filament occurred at -30.0°C. Post-thaw membrane rupture was visible, with cytoplasm extruded into the gaps between, the cell wall/membrane. In some filaments rupture of the cell wall was also evident with visible leakage of intracellular material into the extracellular environment.

Ultrastructural changes in *V. sessilis* were investigated by comparing TEM sections of treated and untreated material fixed immediately post-thaw. Untreated filaments contained many regularly arranged chloroplasts [Fig. 4.10 (1)]. The chloroplasts had an elongated form and contained pyrenoids and parallel-orientated thylakoids, without grana organisation. The inter-thylakoid spaces were narrow [Fig. 4.10 (1, 2)]. Mitochondria often appeared to be associated with chloroplasts and were dense in aspect with cristae occupying their entire volume [Fig. 4.10 (3, 5)]. Nuclei were uniform and dispersed throughout the filament [Fig. 4.10 (4)]. A number of additional structures were observed including: microbodies, endoplasmic reticulum and starch and lipid inclusions [Fig. 4.10 (1,5)].

Cooling and freezing affected the ultrastructural integrity in *V. sessilis*. Supercooling filaments to subzero temperatures (-10°C) resulted in no detectable changes in the organelle form or distribution [Fig. 4.10 (6-10), Table 4.3]. However, significant lipid deposits were observed within the chloroplasts, these were closely associated with thylakoid membranes [Fig. 4.10 (6, 7)]. Cooling until extracellular ice was present at -12°C also resulted in no change in the organelles ultrastructure. However, in some TEM sections a degree of disruption from the expected arrangement of organelles was observed (Table 4.3).

Table 4.3. Effects of cryopreservation on *Vaucheria sessilis*

Treatment[a]	O_2 Evolving Capacity[b]	Ice		TEM[e]	Re-growth[f]
		Ext.[c]	Intra.[d]		
Control[g] (0°C)	100	-	-	N	100
0°C → -10 °C[g,h]	82 ± 4	-	-	N	100
0°C → -12 °C[g,i]	80 ± 3	+	-	N/D	100
0°C → -35 °C[g]	0	+	+	D	0
0°C → -35 °C → -196 °C[g]	0	+	+	D	0
Methanol[j] (0°C)	58 ± 18	-	-	N	100
0°C → -12°C[j,i]	62 ± 22	+	-	N/D	100
0°C → -35°C[j]	0	+	+	D	0
0°C → -35°C → -196°C[j,k]	0	+	+	D	0
-196°C direct[j,l]	0	+	+	D	0

[a] Cooled at -1°C min.$^{-1}$.
[b] Expressed as % of untreated control 48 h. post-thaw.
[c] Extracellular ice present.
[d] Intracellular ice present.
[e] Effect on ultrastructure.
[f] Expressed as % of untreated control.
[g] Filaments incubated at 0°C for 15 min.
[h] Supercooled material.
[i] Supercooled, extracellular ice present.
[j] Filaments incubated for 15 min. in the presence of methanol (10% w/v).
[k] Cooled a -100°C min.$^{-1}$ from -35°C
[l] Plunged into LN, cryoprotectant absent.

+/- Present/Absent
N/D Normal ultrastructure/ Disruption of ultrastructure

n = 3, errors are expressed as standard errors of mean.

After a freeze-thaw cycle to -60°C the filament structure was highly altered with disruption to the distribution and form of organelles [Fig. 4.10 (11-15), Table 4.3]. The mitochondria, endoplasmic reticulum and nuclei were all extensively damaged [Fig. 4.10 (11-15)]. Furthermore, the chloroplasts were distorted into irregular forms, the arrangement of thylakoids was also greatly modified with their stacked, parallel-layered

structure no longer visible and the inner thylakoid spaces much increased [Fig. 4.10 (12)]. Micobodies were broken into smaller bodies and numerous lipid globules were noted in intracellular spaces. Structures of unusual aspect were commonly observed and were assumed to be vesiculated rough endoplasmic reticulum [Fig. 4.10 (15)]. Differentiation between cells cooled to -60 or -196°C was difficult, however, some additional disruption to the organelles was evident at -196°C (Table 4.3). Filaments which were plunged directly into LN (-196°C) had comparable damage to that observed in specimens frozen using two-step protocols (Table 4.3). An additional observation was that increased vesiculation was common in all TEM sections which had undergone intracellular ice nucleation (Table 4.3).

Table 4.4. Effect of cooling rate on intra and extracellular ice nucleation in *Vaucheria sessilis* using two-step cooling

Cooling Rate to -35°C [a]	Extracellular Ice (°C)	Intracellular Ice (°C) [b]	Flashing event (°C) [c]
-20 °C min.$^{-1}$	-12 → -18	-21.2 → -26.2	-30.0 → -30.3
-10 °C min.$^{-1}$	-12 → -18	-21.9 → -30.0	-21.9 → -30.0
-1 °C min.$^{-1}$	-12 → -18	-32.0 → -70.0	-84.0 → -100.0
-0.5 °C min.$^{-1}$	-12 → -18	-116.0 → -122.0	-116.0 → -122.0

[a] Filament held at -35°C for 30 min. followed by cooling at -100°C min.$^{-1}$ to -180°C.
[b] Darkening of the filament associated with the presence of intracellular ice.
[c] Intracellular ice front running the length of the filament.
n = 3

4.3.4 *Enteromorpha intestinalis*

E. intestinalis was also frozen following a two-step protocol (see 3.2) which yielded 100% viability post-thaw. Some cells within each filament were observed to undergo

intracellular ice nucleation, however, this did not promote general nucleation of intracellular ice throughout the filament (Table 4.5).

Table 4.5 Effect of cooling rate on *Enteromorpha intestinalis*

Cooling rate [a]	Intracellular ice [b]	% [c]	Temp (°C) [d]
-10	+	15	-62
-1	+	2	-100

[a] Controlled cooling rate to -35°C (°C $min.^{-1}$), and at -100°C $min.^{-1}$ to -170°C.
[b] Intracellular ice observed
[c] % of cells displaying intracellular ice
[d] Temperature of intracellular ice nucleation event
\+ Event observed
\- No event observed
n = 3

4.4 Discussion

Studies have highlighted a number of visible chilling and freezing injury responses in the algae examined. Light cryomicroscopy proved to be an effective approach for the visualisation of post-treatment events associated with chilling and freezing. Cryomicroscopy allowed changes due to chilling and freezing to be observed "real time" during simulated cryopreservation protocols (Fleck *et al.*, 1997a).

4.4.1 Cryomicroscopy

The technique has previously been employed to identify significant causes of lethal freeze-induced injury in microalgae. These studies include assessments of: *Chlamydomonas* (Roberts *et al.*, 1987), desmids (Morris *et al.*, 1986), the filamentous alga *Spirogyra* (Morris & McGrath, 1981), the marine alga *Tetraselmis suecica* (Day *et al.*, in press) and *V. sessilis* (Fleck *et al.*, 1997a). In most cases, loss of viability has been attributed to events associated with intracellular ice formation. Furthermore, cryomicroscopy has been used to develop successful cryopreservation protocols,

avoiding lethal freeze-induced injury, for yeasts (Morris *et al.*, 1988a) and filamentous fungi (Smith *et al.*, 1986).

However, the potential value of cryomicroscopy is dependent upon the accurate duplication of the cooling regimes experienced by samples being cryopreserved. Controlled cooling in a programmable freezer assumes that the internal temperature is uniform throughout the chamber and therefore allows for the possibility of supercooling the sample. Initial investigations using the Planer CM3 cryostage, suggested that intracellular ice was not a common cause of cell injury in *V. sessilis* or *E. gracilis*. In this system extracellular ice nucleation occurred at high subzero temperatures (0 to -2°C). This appeared to be due to "seeding" of the extracellular solution which acted as a point of ice-nucleation. This "seeding" effect was caused by the temperature gradient present on the Planer CM3 cryostage. However, without monitoring for thermal events associated with ice nucleation, for example using a differential scanning calorimeter, it would be impossible to rule out the occurrence of intracellular ice in these experiments. In a previous cryomicroscopic study on *Spirogyra* (which is approximately the same size as *Vaucheria*), using a similar stage, "flashing" was not reported, but large intracellular ice crystals were observed in all filaments (Morris & McGrath, 1981). The design of the Linkam BCS 196 cryostage allowed the cryostage and specimen to be cooled at controlled rates to -170°C. This, facilitated the study of specimens at temperatures commonly achieved during cryopreservation and reduced the possibility of artifacts being generated by excessive ice crystal growth due to the formation of more stable hexagonal ice forms (I_h) at high subzero temperatures (1.9.2).The Linkam cryostage was used without mechanical ice seeding, thus providing a uniform temperature across the entire cooling-block, allowing supercooling and ice-nucleation of the extracellular solution to be visualised. In addition, it was possible for intracellular "flashing" events to be observed in *V. sessilis* (Fleck *et al.*, 1997a).

The development of techniques which allowed thin, unicellular, samples to be mounted on the cryostage permitted more detailed observation of intracellular ice events on the cells (Fleck *et al.*, 1997a). "Thinner" specimens, mounted in sealed chambers, prevented evaporation of the medium which could have resulted in the cells being exposed to additional cumulative osmotic changes, and/or dehydration, in addition, to those

encountered during a "normal" cryopreservation protocol (Fleck *et al.*, 1997a). Although most intra- and extracellular events were readily observed with standard bright field microscopy a number of alternative microscopic techniques allowed more detailed observation of visible events. Both Phase-contrast and DIC microscopy techniques proved effective in the examination of chilling and freezing injury (Fleck *et al.*, 1997a) [Figs. 4.2, 4.7 (A-D), 4.9 (A-D)]. Fluorescence microscopy, was used to allow clearer observations of the filament in the frozen environment, with less distortion of the image by extracellular ice (Fleck *et al.*, 1997a).

Following these studies, several areas of potential cryoinjury were noted including: excessive cellular dehydration/cryodehydration [Figs. 4.9 (A-D)], mechanical disruption of cells by extracellular ice crushing [Figs. 4.9 (C,D)], intracellular ice nucleation on supercooling [Fig. 4.9 (C), Tables 4.1, 4.2, 4.4, 4.5], gross post-thaw disruption of intracellular architecture and cell membrane rupture [Figs. 4.9 (D)].

The detection of intracellular ice in *V. sessilis* at all cooling rates employed was likely to be an indicator of lethal freeze injury (Tables 4.3, 4.4). This association between intracellular ice formation and lethal freeze injury has previously been observed in plant systems, where there may be loss of compartmentalisation when growing intracellular ice crystals rupture cellular membranes (Levitt, 1980). The formation of intracellular ice in *V. sessilis* close to the point of extracellular ice nucleation, indicated that only a limited amount of further supercooling of cellular water occurred before secondary, intracellular freezing (1.9.2). In the absence of heterogeneous ice-nuclei, cellular water could be expected to supercool to the homogeneous ice-nucleation temperature (-38°C) before freezing (Ashworth, 1996).

In cells cooled at rapid, suboptimum rates, intracellular ice nucleation was observed (Tables 4.1, 4.2, 4.4, 4.5). Indicating insufficient concentration of the cytosol had occurred in order to preclude intracellular ice nucleation, under these conditions lethal injury could be attributed to intracellular ice nucleation due to insufficient cryodehydration caused by too rapid cooling (Steponkus *et al.*, 1992, Mazur, 1970). However, the temperature of intracellular ice nucleation was dependant upon cooling rate indicating that cryodehydration was occurring, concentrating the cytosol and

influencing its freezing point (Tables 4.1, 4.2, 4.5). The principles of cryodehydration are discussed in detail in 1.9.2-1.9.3.

However, cryoprotectant toxicity and the period of exposure to increased salt concentrations may also be significant causes of freezing injury and subsequent death (Pegg, 1987) and are categorised as secondary freeze-induced injury and cryoprotectant toxicity (Levitt, 1980). Cryoprotectant toxicity has been shown to effect cellular viability in Chapter 3. In addition, during two-step cryopreservation protocols, the cells are subjected to osmotic changes with potentially damaging increases in extracellular salt concentration during freezing. In this study *V. sessilis*, *E. gracilis* and *H. pluvialis* were subjected to osmotic changes and underwent reduction in volume whilst in the frozen environment due to cryodehydration (50 ± 2%, >40% and 72% ± 0.5% respectively of their original volume). These events are comparable of observations of cell shrinkage in *Penicillium expansum* hyphae during freezing and thawing by Coulson *et al.* (1986). If not fully mitigated by cryoprotectants, the resulting rise in salt concentration may potentially damage the filament. In studies by Morris (1981), increasing molarity of NaCl in unfrozen water at various subzero temperatures was monitored. In water lacking cryoprotectant the molarity of the unfrozen solution increased from 0.15 M to 5 M by -30°C, however, in water containing 3.0 M glycerol the molarity of the unfrozen solution reached a plateau at < 1 M) (Morris, 1981). The absence of intracellular ice nucleation in *E. gracilis* when cells were cooled at -0.3°C $min.^{-1}$ points to excessive cryodehydration as the probable mode of lethal injury as determined by post-thaw viability studies discussed in Chapter 3.

Cryoprotectant exposure also affected the photosynthetic capacity of *V. sessilis*, filaments were, however, able to recover after being removed from the cryoprotectant (Table 4.3). The influence of the cryoprotectant solution on photosynthetic capacity of *E. gracilis* has been discussed in detail in Chapter 3. In related studies, increasing the concentration of cryoprotectant and lengthening exposure times have been demonstrated to affect viability (Fleck, unpublished data). The addition of cryoprotectant is, however, necessary because it acts to mitigate injury encountered during freeze-induced dehydration (Steponkus *et al.*, 1992). Furthermore, it protects cells by modulating rises in solute concentration during freezing (Pegg, 1987).

In all cryomicroscopic studies presented here, the lack of immediate nucleation of intracellular ice in cells which were in a frozen external environment indicated that the cell wall/membrane was able to act as an effective barrier to external ice, preventing intracellular ice nucleation and allowing further supercooling of the cytosol and cryodehydration, a key component of any two-step cryoprotocol. The ability of membranes to block intracellular ice nucleation to -15°C and the ability of cells to supercool, even when external ice is present, indicated in Mazurs' (1970) systems the absence of effective ice nucleators within the cells interior and the ability of the cell membrane to block nucleation (Mazur, 1970). Comparable observations of supercooled intracellular material, indicting the absence of effective ice nucleates, were seen in the algae investigated (Tables 4.1, 4.2, 4.4, 4.5).

Membrane damage is a further manifestation of freezing stress and disruption of the plasma membrane has been considered a primary cause of freezing injury (Benson, 1990) (1.11-1.11.1.1). Injury may be observed as lysis of the cell or protoplast, leakage of electrolytes and other cell constituents and breakdown of fine structure (Singh and Miller, 1985). Although loss of functional bilayers can result in diminished membrane material and lysis on thawing (Wiest & Steponkus, 1978), damage occurring in the frozen environment cannot be precluded. In support, loss of compartmentalisation of material and degradation of phospholipids by lytic enzymes in the frozen state has been described (Stout *et al.*, 1980; Rajashekar *et al.*, 1979; Yoshida, 1979a,b). These studies have shown that lethal membrane alterations can occur during freezing in the extracellularly frozen cell and have pointed to the disruption of membrane compartmentalisation, structure and function at lethal freezing temperatures (Singh and Miller, 1985). The disruption of membranes and cellular compartmentalisation is likely when cells are exposed to intracellular ice nucleation events or excessive cryodehydration and may be further visualised by employing TEM.

Chloroplasts damaged by low temperatures and freezing also exhibit a number of symptoms (Garber, 1979). Ultrastructural studies have shown a range of damage related symptoms including: swelling and distortion of the thylakoids, formation of small vesicles at the envelope, accumulation of lipid droplets and disintegration of the

envelope (Wise *et al.*, 1983). In studies at 5°C symptoms were time dependant with the time course being related to chilling injury (Wise *et al.*, 1983). Freezing of thylakoid membranes causes inactivation of phosphorylation (Heber *et al.*, 1981). Cryoprotective compounds which protect intact cells against freezing also protect thylakoid membranes against inactivation of phosphorylation (Heber *et al.*, 1981). This indicates that the thylakoid membranes may be part of the membrane systems which are responsible for the sensitivity of some cells to cold. Mechanical stress produced by external growth of ice crystals has largely been discounted as a mode of injury in cryopreserved tissues (Heber *et al.*, 1981). Inactivation of thylakoid membranes by freezing is more extensive the higher the concentration of solute and differ from the kinetics of inactivation due to ice formation (Heber *et al.*, 1981). If the temperature during freezing reaches the eutectic of the system and if there is no barrier to solute crystallisation, complete solidification will occur, under these conditions mechanical effects produced by crystal growth may contribute to inactivation.

4.4.2 Transmission electron microscopy studies

Use of transmission electron microscopy allowed ultrastructural changes caused by the cryopreservation protocol to be investigated. Results indicated a strong correlation between the presence of intracellular ice and gross disruption of major organelles including; mitochondria, nuclei, chloroplasts and endoplasmic reticulum [Fig. 4.10 (6-15), Table 4.3). In all *V. sessilis* sections examined where intracellular ice had occurred, no normal organelles were observed. In comparison all organelles appeared normal and were easily identified in control and undamaged sections. Thylakoid parallel-orientation, was lost and inter-thylakoid spaces are increased, in freeze-thaw damaged samples. Additionally, increased intracellular vesiculation and disruption/change in the form of the microbodies was also characteristic of filaments which had undergone intracellular ice nucleation. Damage may be attributed to expansion of the water in the cytosol/organelles during freezing. TEM sections obtained from *E. gracilis* cells fixed after exposure to various stages in a cryopreservation protocol did not display the accumulation of lipid droplets in the chloroplasts at high subzero temperatures that were observed in *V. sessilis*. However, in cells cooled to lower temperatures -60°C and LN, increased vesiculation was observed which was commonly associated with starch and/or

lipid deposits. The increased vesiculation and displacement of cytoplasm in *E. gracilis* may be due to a number of factors. These include the possibility of localised intracellular ice, possibly within vacuoles, disruption or changes in the distribution and/or composition of storage deposits, or the vesiculation may be due the effects of cryodehydration. In addition, in all sections containing cells which had been plunged directly into LN and a proportion which had been two-step cooled and plunged into LN, disruption of the cytoplasm, comparable to that observed in sections of *V. sessilis* which had experienced intracellular ice nucleation was observed including: disruption of the mitochondria, nucleus and chloroplast form.

The ultrastructural modifications were consistent with those observed in freeze-thaw *Undaria pinnatifida* gametophytes (Ginsburger-Vogel. *et al.*, 1992) who reported that in cultures where post-thaw viability was low, the structure of most cells was highly altered and the arrangement of thylakoid membranes considerably modified. In studies of the effect of osmotic stress on *Chlamydomonas reinhardtii* Morris *et al.* (1985) reported that following osmotic shrinkage and rehydration, fragmentation of the mitochondrial network was observed within potentially viable cells. Morris *et al.* (1985) reported that mitochondria reacted differently to osmotic stress at 20°C than to dehydration stress induced during freezing. However, the damage observed indicated mitochondria may be sensitive to cryoinjury in freeze-recalcitrant organisms (Fleck *et al.*, 1997a).

4.4.3 Specific cellular responses

E. gracilis underwent a series of characteristic changes in cell morphology (loss of elongate form) and loss of flagellum on cooling to 0°C indicated that the cells may be experiencing stress (Fig. 4.2). Although both responses are common in stressed *E. gracilis* cells they are not generally lethal. However, the responses do indicate that *E. gracilis* was experiencing stress due to cooling and/or cryoprotectant toxicity and that the flagellar insertion point may be a potential point of weakness in the cell. No cells with attached flagella were observed immediately after thawing.

V. sessilis was observed to experience a series of additional injurious events, due to other freeze-induced stresses these included: cellular damage through the disruption of intracellular organelles by extracellular ice crushing and the "turbulent" displacement and mixing of organelles between -17.4 and -26°C. The "turbulent" displacement and mixing of organelles (-17.4 and -26°C) occurred a few degrees below extracellular ice nucleation (-12°C). This observation may explain the absence of any major or permanent damage to filaments cooled to -12°C (Fig. 4.11, Table 4.3). However, the damaging effects of "ice crushing" may have some bearing on the loss of viability in filaments cooled to lower subzero temperatures (Table 4.3).

A further problem in the cryopreservation of *V. sessilis*, highlighted by TEM studies was the variable nature of the filaments cell wall [Fig. 4.10 (1,6,11)]. It was probable that cell morphology made uniform dehydration of the filament difficult to achieve. In non-coenocytic organisms insufficient dehydrated of the cytosol in localised areas would not necessarily be a problem, as each cell would be effectively isolated from other cells. In the filamentous alga *Enteromorpha intestinalis* (L.) Link, CCAP 320/1 the author has observed (using cryomicroscopy) (Table 4.5), that the presence of intracellular ice in a proportion of the cells, was not necessarily lethal, and the alga was able to recover fully. Further cryomicroscopic studies demonstrated that by increasing the cooling rate (-1°C $min.^{-1}$ → -10°C $min.^{-1}$) the number of cells undergoing intracellular ice nucleation increased. Employing rapid cooling techniques, where a higher proportion of cells were likely to experience intracellular ice nucleation, still allowed regeneration of a healthy alga, however, recovery times lengthened. Studies of intracellular ice formation in various species, using cooling rates which result in intracellular ice in 50 % of cells corresponded to cooling rates which result in 50 % cell survival in bulk freezing experiments (reviewed by Toner, 1993). However, in coenocytic *V. sessilis*, the observation of ice fronts running the length of the filament presents a particular problem, with any single point of intracellular ice nucleation potentially able to cause ice formation throughout the filament and ultimately resulted in total loss of viability. *V. sessilis* was also probably more sensitive to damage through external ice crushing, due to the lack of cell compartmentalisation presenting no natural barriers to intracellular ice fronts. These additional problems which are due to the morphology of *V. sessilis* are

likely to make the development of a cryopreservation protocol extremely difficult and contribute to the present freeze-recalcitrance of *V. sessilis*.

4.4.4 Future approaches which could be employed to investigate cryoinjury

New approaches for the visualisation of cellular damage and recovery in cryopreserved material are likely to be closely related to current advances in the field of microscopy. New generations of high-resolution, field-emission, scanning electron microscopes (SEM) are now capable of producing images of the surfaces of biological specimens that rival, in terms of resolution and contrast, those produced by conventional transmission electron microscopy (TEM) (Pawley, 1997). In addition, variable vacuum technology now permits the study of partially hydrated specimens, avoiding the requirement for complex dehydration procedures which may in themselves introduce artefacts. This offers considerable scope for the future investigation of cryoinjury at high resolution. Furthermore, techniques including cryofixation and freeze fracture of specimens also offer the ability to visually investigate intracellular events including organelle disruption and damage.

The recent advent of confocal microscopy; an advancement of light microscopy which employs laser, computer, and imaging technologies, permits "optical sectioning" of specimens rather than mechanical sectioning (by cutting) of both thin and relatively thick microscope specimens (Paddock, 1996). Confocal microscopy is capable of being used in both fluorescence and reflection imaging modes and the resulting images have far superior resolution and contrast than those obtained using a conventional light microscope. The capability is achieved by using a monochromatic laser light in a scanning raster across a specimen. When a confocal pinhole is placed in the light path between the sample and the detector, most out-of-focus light is removed. Digitised confocal images may be further analysed or improved by employing image processing and 3-D reconstruction (Czymmek *et al.*, 1994; Shotton, 1995). The confocal microscope is therefore a particularly valuable research tool for imaging fluorescently labelled biological specimens. (Paddock, 1996; Cox, 1993; Paddock, 1994; Petroll *et al.*, 1994). Due to its ability to image relatively thick sections, marrying of confocal microscopy with a cryostage offers considerable scope for the further investigation of

cryoinjurious events during a cryopreservation protocol. Furthermore, advances in video capture, digital cameras and digital image analysis have the possibility of radically improving the study of cryoinjury.

Microscopy has also been adapted to the study of antioxidants and oxidative stress including the extremely toxic hydroxyl radical and the antioxidant enzyme superoxide dismutase (1.11.2-1.11.5.1). The antioxidant enzyme superoxide dismutase (SOD) converts superoxide radicals into hydrogen peroxide and molecular oxygen and therefore represents a primary defence against oxidative damage (1.11.2-1.11.5.1). Immunogold labelling has been applied to investigate the role of desferrioxamine in the scavenging of hydroxyl radical on disruption of the blood brain barrier in guinea pigs (Guy *et al.*, 1994) (1.11.9). Furthermore, immunogold-labelling has been applied to determine the distribution of superoxide dismutase in Spinach leaves. In Spinach leaves fixed by a rapid freeze/substitution method which allowed visualisation of intact chloroplasts, embedded sections were immunogold-labelled with an antibody against CuZn-SOD, permitting the local concentrations of CuZn-SOD on the stroma-facing surfaces of the thylakoid membranes to be estimated (Ogawa *et al.*, 1995). By employing antibodies against *Anabaena cylindrica* Fe-SOD, the focal point of the enzyme could also be identified and by measuring the levels of Fe-SOD labelling changes in Fe-SOD levels at different photon irradiances could be determined (Caiola & Canini, 1993). Further related studies, determining the focus of SOD in cells have been reported (Caiola & Canini, 1994; Canini *et al.*, 1992). The principles of immunogold-labelling have also been discussed in detail by Hayat (1989; 1991).

These techniques together with current advances in fluorescent labelling techniques offer considerable scope for the future investigation of mechanisms of cell damage and recovery through the use of visual techniques (Haugland, 1996). Further optical methods for the investigation of cryoinjury are discussed in Chapter 5, in which flow cytometry and fluorescent vital stains have been employed.

4.4.5 Conclusions

Microscopy is clearly an effective technique for the study of cryoinjury and damage. However, it has clear advantages and disadvantages for the investigation of the mechanisms of cryoinjury. The technique has considerable potential, particularly as a tool for developing non-damaging protocols for nominally freeze-recalcitrant strains. However, it can only provide part of the answer to the question of how and where damage occurs and may prove most useful when used in conjunction with additional investigative techniques. In particular it may be used to highlight potential areas for further investigation including: lethal and non-lethal stress, intracellular ice formation and the effects of excessive and insufficient cryodehydration. Many of these areas of potential cryoinjury have been investigated in subsequent chapters. Chapter 5 considers the effect of cooling rate on cellular injury, either through excessive cryodehydration or insufficient cryodehydration. Chapters 7 and 8 consider oxidative stresses within the organisms and their ability to mitigate oxidative injury.

Chapter 5.

An investigation of cellular damage and recovery.

Contents **Page No.**

5.1 Introduction

In chapter 4 a series of markers were identified, as being indicative of both lethal and non-lethal injury in *Euglena gracilis* (Fig. 4.2). In addition, a number of events culminating in cell mortality were observed in *Euglena gracilis, Haematococcus pluvialis* and *Vaucheria sessilis*. These events were attributed to extremes of either excessive cryodehydration or insufficient cryodehydration (Tables 4.1, 4.2, 4.3, 4.4, 4.5). Furthermore, cryoprotectant toxicity has been identified as a potentially lethal source of injury in algae (Chapter 3).

5.1.1 Objectives

This study aimed to employ flow cytometry in conjunction with vital staining to investigate cryoinjury in algae. Furthermore, changes in cellular photosynthetic capacity will be investigated and it is hoped that this will permit the identification of lethal and non-lethal stresses due to stages of a cryopreservation protocol.

5.1.2 Effects of cryopreservation on metabolism

In frozen cellular material mortality has often been attributed to intracellular ice formation, excessive cryodehydration and/or cryoprotectant toxicity (See Chapters 3 & 4). Furthermore, cryoprotectant exposure, cooling to 0°C or subambient temperatures (in the absence of ice) have been demonstrated to be non-lethal for *E. gracilis* and *V. sessilis* (Fig. 3.5, Table 4.3). However, the possibility of reversible/non-lethal stress cannot be precluded. In addition, further factors including: cell lysis, modifications at the cell membrane level, breakdown of fine structures and/or loss of cellular compartmentalisation have all been proposed as possible mechanisms of freezing injury and are discussed in detail in 1.11-1.11.1.2. Theories of cellular damage include the loss of functional bilayers which may result in diminished membrane material and cell lysis on thawing (Wiest & Steponkus, 1978). In addition, damage may also occur whilst the cell is in the frozen environment (1.11.1). It has been hypothesised that loss of cellular compartmentalisation during freezing may induce the degradation of phospholipids by phospholipase (Stout *et al.*, 1980; Rajashekar *et al.*, 1979, Yoshida, 1979a,b) (1.11.1).

These investigations target modes of lethal injury incurred due to exposure to a frozen environment. In addition, injuries, both lethal and non-lethal which occurs during the cryopreservation protocol will be studied.

5.1.3 Use of the oxygen electrode

Measurement of oxygen evolution and respiration in a closed system permits the determination of photosynthetic/respiration rates. This allowed monitoring of the post-treatment photosynthetic capacity of an alga. In addition, by assaying photosynthetic capacity throughout the recovery period, non-lethal/reversible, but metabolically inhibitory, responses may be identified (by measuring inhibition of photosynthetic capacity). Furthermore, photosynthetic capacity in the algae may be employed to determine viability levels.

5.1.4 Use of the flow cytometer

Vital staining with fluorescein diacetate (FDA) may be employed to assess post-exposure viability levels in cells (Harding & Benson, 1995). Fluorescing cells and non-fluorescing cells may be counted, with the aid of a microscope, on a haemocytometer slide to determine the % viability. However, this technique has a number of limitations including: subjective interpretation of positive/negative staining by the microscopist and the comparatively low numbers of cells viewed.

Flow cytometry is a rapid, highly sensitive technique which may be employed for multiparametric analysis (*e.g.*, quantification of antigen expression of cell surface antigens) and cell sorting (Maftah *et al.*, 1993; Drouet & Lees, 1993). It may be employed as a technique for measuring many cell functions including: the determination of cell viability, intracellular calcium and pH levels, membrane potential, enzyme activity, membrane fluidity and endocytosis (Maftah *et al.*, 1993). In addition, the facility to rapidly analyse extremely large numbers (>1000 cells) gives flow cytometry an important advantage over conventional microscopical techniques (2.5.3), particularly

when employed as an assessment of viability (haemocytometer cell counts would examine, at most, a few hundred cells, flow cytometry will analyse thousands).

Recent trends in flow cytometry have allowed new techniques for the quantification of microbial population size and activity to be developed (Porter *et al.*, 1996). These have been complimented by advances in fluorescent dye technology which now offer probes for a variety of cellular functions (Porter *et al.*, 1996; Jansson & Prosser, 1997; Loken, 1990; Haugland, 1996). In bacteriology, flow cytometry has found use in viability analysis, investigation of stress induced changes, specific cell detection and the sorting of cells, *e.g.*, screening for eukaryotic water-borne pathogens (Porter *et al.*, 1996; 1997). The ability to rapidly perform viability assessments on large numbers of cells and monitor for stress induced changes (morphological change) also has potential applications within the field of cryobiology, where microorganisms or cell cultures are being studied. Algal viability may be determined by vital staining post-treatment and assessed rapidly by flow cytometry. In addition, the ability to quantifying and accurately measure volume changes as changes in the forward light scatter [the forward scatter height (FSH)] permits changes in cell size due to cryoinjury to be detected. These effects of cryopreservation were previously highlighted as a possible marker of cryoinjury in Chapter 4 (Figs. 4.3, 4.8).

5.2 Materials and methods

5.2.1 Organisms and culture regimes

Cultures selected for study are detailed in 2.1. (*Haematococcus pluvialis* Flotow CCAP 34/8, *Euglena gracilis* Klebs CCAP 1224/5Z and *Vaucheria sessilis* (Vaucher) De Candolle ex Collins CCAP 745/1C). Filaments of the xanthophytic alga *Vaucheria sessilis* were prepared as described in 4.2.1. Organism culture regimes and recovery conditions were as described in 2.2-2.3.

5.2.2 Cryopreservation procedures

Cryoprotectant solutions were prepared and used as described in 4.2.2. Vials containing 0.5ml of algae/cryoprotectant were frozen by controlled to their intermediate holding temperature using a Planer Kryo 10 programmable freezer (Planer, UK) (2.4). Cooling rates of -20°C $min.^{-1}$, -10°C $min.^{-1}$, -1°C $min.^{-1}$, -0.5°C $min.^{-1}$, and -0.3°C $min.^{-1}$ were employed. Vials were cooled to -35°C or -60°C and held at their intermediate temperature for either 15 or 30 min. periods, prior to being plunged directly into LN.

Vials were thawed using either a one or a two-step protocol. One step protocols employed a single direct immersion in a pre-heated water bath at 40°C. Two-step protocols employed a method by which vials were first allowed to slowly warm while being held in the air for 1 min. followed by rapid warming in a pre-heated 40°C water bath.

In addition, specific studies on the effect of warming procedures on *E. gracilis* employed a further one-step protocol where vials were thawed following a controlled warming rate of +100°C $min.^{-1}$ in a programmable freezer to 25°C or a further two-step protocol where cells were initially placed in a programmable freezer pre-cooled to -130°C and held for 30 min. prior to being directly immersed in a pre-heated 40°C water bath. A specific study on the effect of osmotic stress was performed on *E. gracilis* which involved exposing cells for 15 min. periods to solutions (0M, 0.175M, 0.25M, 0.5M, 1M, 2M, 4M) of sodium chloride (NaCl) prepared in deionised water and recovered in EG:JM medium for 48 h.

All vials were agitated in the water bath until the last ice crystals had melted as detailed by Day *et al.* (1997). The thawed material was then aseptically transferred to appropriate fresh, sterile, medium (2.2), and used in the viability assays as outlined in 2.5. All errors are expressed as standard errors of mean.

5.2.3 Flow cytometry

Flow cytometry was carried out on cells after a recovery period of either 24 h. or 48 h. using a FACStar Plus flow cytometer (Becton Dickinson, UK) as described in 2.5.8, using the vital fluorescent stain fluorescein diacetate (FDA) prepared in methanol (2.5.2.1). Positive fluorescent staining may be attributed to intact cell membranes and active enzymes [functioning esterase activity (2.5.2.1)].

Prior to employing flow cytometry, preliminary investigations were performed with the aid of a haemocytometer slide which enabled the proportion of viable stained cells and non-viable cells to be determined (2.6.1). In addition, the duration of recovery during which "false positive" stained cells could be detected was determined (2.6.1).

5.2.4 Oxygen electrode and chlorophyll measurements

5.2.4.1 Chlorophyll extraction and measurement

A known wet weight of sample was heated, in the dark, at 80°C in 3 ml methanol for 20 min., chlorophyll levels were then determined as described by MacKinney (1941) (2.5.6).

5.2.4.2 Photosynthetic capacity

Respiration and oxygen evolution rates were measured using a Rank oxygen electrode according to Whitelam and Codd (1983). Illumination was constant and non-limiting for oxygen evolution steps (440 μmol m^2 s^{-1}) (2.5.7).

5.3 Results

5.3.1 *Euglena gracilis*

The effect of both cooling and thawing regimes were investigated for *E. gracilis* with the aid of flow cytometry.

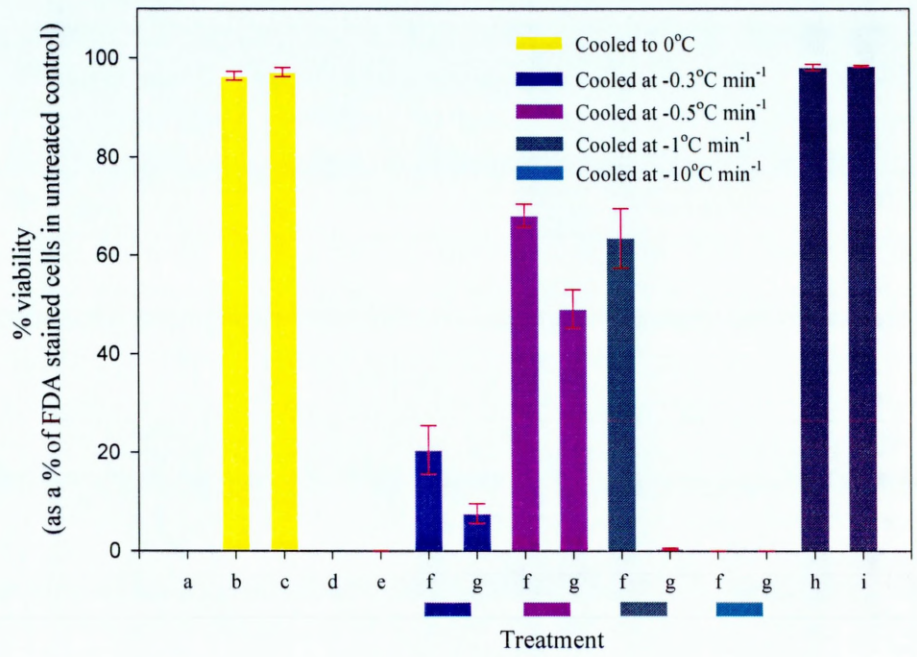


Figure 5.1 The effect of different cooling rates and supercooling on post-treatment viability in *Euglena gracilis*, assessed by FDA staining 48 h. after treatment.

(a) Unstained untreated control, (b) Untreated control, (c) Cells exposed to cryoprotectant at 0°C (10 % (v/v) methanol) for 15 min., (d) Cells plunged directly into LN, no cryoprotectant present, (e) Cells plunged directly into LN, no cryoprotectant present, unstained, (f) Cells control cooled to -60°C and held for 30min, (g) Cells plunged into LN from -60°C two-step thaw 1min in air followed by immersion in a 40°C water bath, (h) Cells supercooled to -10°C, cryoprotectant present, (i) Cells supercooled to -10°C, without cryoprotectant. All material was thawed using a simple two-step protocol.
n = 3, errors are expressed as standard errors of mean.

Different controlled cooling rates affected the post-thaw viability of *E. gracilis* after exposure to both the intermediate holding temperature (-60°C) and LN. Cooling at rates > -1°C min.$^{-1}$ to -60°C resulted in 100% mortality in cells thawed after exposure to -60°C and LN [Fig 5.1 (checked blue; f, g)]. Cooling cells at -1°C min.$^{-1}$ to -60°C resulted in viability levels of 63% ± 6% after exposure -60°C, however no viable cells

were obtained after exposure to LN [Fig. 5.1 (dark blue; f, g)]. Employing the optimum cooling rate of -0.5°C min.$^{-1}$ to -60°C permitted the recovery of viable cells after exposure to both -60°C and LN, viability levels of 68% ± 2% and 49% ± 4% respectively were obtained [Fig. 5.1 (magenta; f.g)]. The previously published method employed a cooling rate of -0.3°C min.$^{-1}$ to -60°C (Morris & Canning 1978) resulted in reduced post-thaw levels of viable cells after exposure to both -60°C and LN 21% ± 5% and 8% ± 2% respectively [Fig. 5.1 (light blue; f,g)]. However, the duration of exposure (15 min. at 0°C) to the cryoprotectant solution [methanol 10% (v/v)] resulted in no significant reduction in post-exposure viability [Fig. 5.1 (b, c)]. In addition, supercooling cells to subzero temperatures in the absence of external ice (-10°C), with cryoprotectant [methanol 10% (v/v)] either present or absent resulted in no significant reduction in post-exposure viability levels [Figs. 5.1 (h, i), 5.2 (k, l)]. For all cooling rates, where viable cells were observed after exposure to LN, optimum viability levels were achieved using a two-step thawing procedure. In cells thawed using a single step thawing protocol after cooling at -1°C min.$^{-1}$ and -0.3°C min.$^{-1}$ post-LN exposure, viability levels were reduced to 0% and 5% ± 1% respectively (Chapter 3). The above data strongly suggested, that viability of *E. gracilis*, following LN exposure could be influenced by the thawing procedure. Slow one-step warming to ambient temperatures at a rate of +100°C min.$^{-1}$ and single-step rapid thawing in a 40°C water bath were both suboptimal with post-thaw viability levels of only 26% ± 5% and 5% ± 1% respectively [Fig. 5.2 (i, j)]. Optimum viability levels for cells recovered from LN were obtained by employing either a simple two-step thawing procedure where cells were first allowed to slowly warm in the air for 1 min. followed by more rapid warming in a pre-heated 40°C water bath, or by initially placing cells in a programmable freezer pre-cooled to -130°C for 30 min. prior rapidly thawing them a pre-heated 40°C water bath. Viability levels for cells thawed after exposure to LN using these two-step protocols were 65% ± 0.5% and 64% ± 4% respectively [Fig. 5.2 (g, h)].

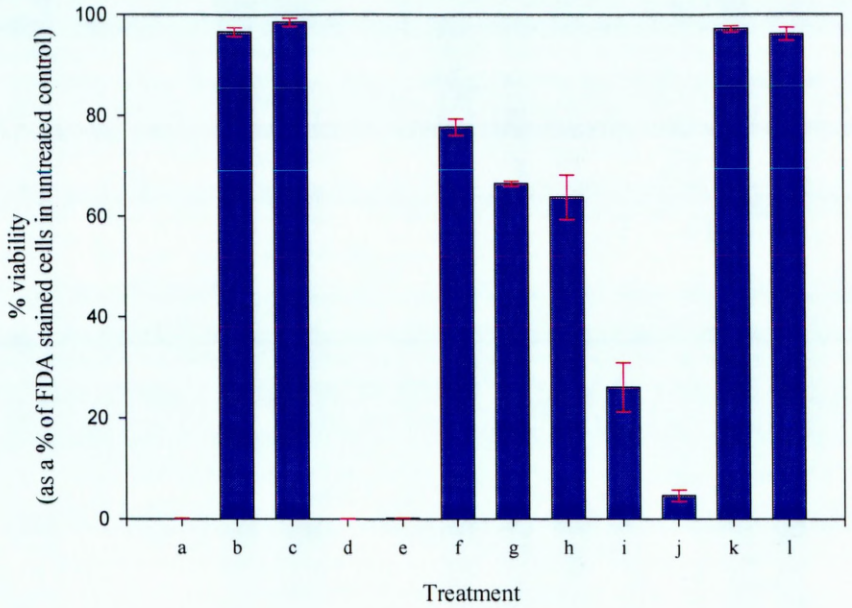


Figure 5.2 The effect of different thawing procedures following a LN plunge on cell viability in *Euglena gracilis* as assessed by FDA staining 48 h. after treatment.

(a) Unstained untreated control, (b) untreated control, (c) exposed to cryoprotectant at 0°C (10 % (v/v) methanol) for 15 min., (d) plunged directly into LN, without cryoprotectant, (e) Unstained, plunged directly into LN, without cryoprotectant, (f) control cooled at -0.5°C $min.^{-1}$ to -60°C and held for 30min; (g) plunged into LN from -60°C and thawed following a simple two-step protocol, 1min in air followed by immersion in a 40°C water bath, (h) plunged into LN from -60°C two-step controlled thaw to -130°C followed by immersion in a 40°C water bath, (i) plunged into LN from -60°C one-step controlled thaw at 100°C $min.^{-1}$, (j) plunged into LN from -60°C one-step thaw in a 40°C water bath, (k) Supercooled to -10°C, cryoprotectant present, (l) Supercooled to -10°C, without cryoprotectant.
n = 3, errors are expressed as standard errors of mean.

Flow cytometry was also employed to investigate the effect of osmotic shock on *E. gracilis*. By exposing *E. gracilis* to varying concentrations of sodium chloride (NaCl) for 15 min. it was found that the highest concentration of NaCl which *E. gracilis* could tolerate without a reduction in post-exposure viability was 0.5M NaCl [Fig. 5.3 (f)]. In cells exposed to concentrations greater than this, lethal injury was observed. In cells exposed to 1M NaCl post-exposure viability was reduced to 34% ± 1%. Exposure to higher concentrations of NaCl resulted in 100% mortality [Fig. 5.3 (d, e)].

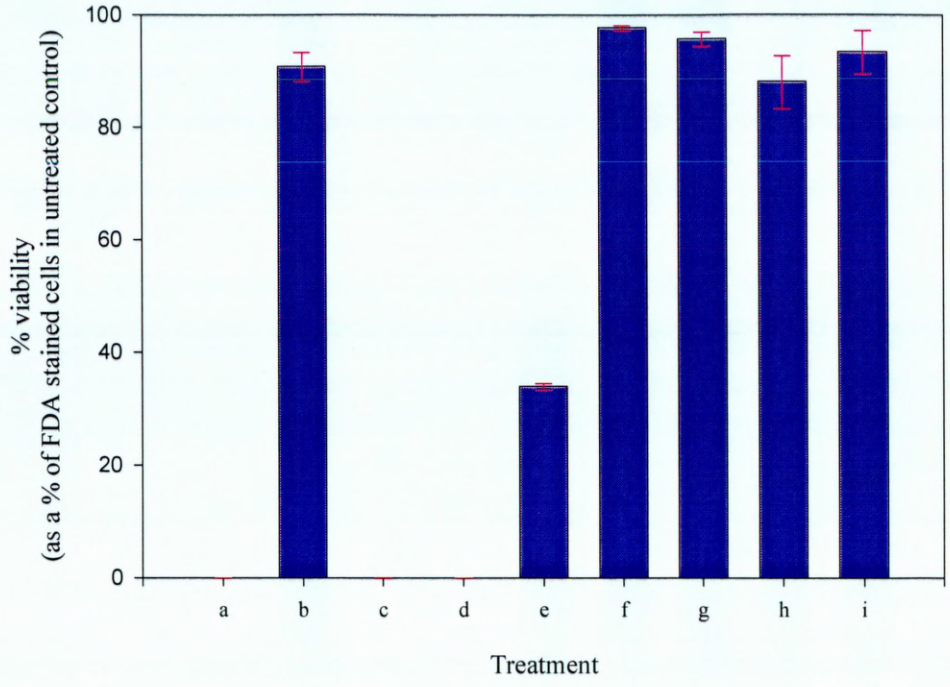


Figure 5.3 Effect of 15 min. exposure to different concentrations of NaCl on the viability of *Euglena gracilis* as determined by FDA staining 48 h. after treatment.

(a) Unstained untreated control, (b) Untreated control, (c) Cells exposed to 4M NaCl, (d) Cells exposed to 2M NaCl, (e) Cells exposed to 1M NaCl, (f) Cells exposed to 0.5M NaCl, (g) Cells exposed to 0.25M NaCl, (h) Cells exposed to 0.175M NaCl, (i) Cells held in deionised water.
n = 3, errors are expressed as standard errors of mean.

Change in cell size was determined by flow cytometry 24 h. and 48 h. after treatments which compared exposure to optimum (-0.5°C $min.^{-1}$ to -60°C) and suboptimal cooling regimes (-0.3°C $min.^{-1}$ to -60°C) and after different degrees of osmotic shock (Fig. 5.4). In cells which had been exposed to potentially lethal events during either a suboptimal cooling protocol, or exposed to osmotic stress, an increase in cell size was detected [Fig. 5.5 (i-n)]. After a 24 h. recovery period cell viability after cooling at -0.3°C $min.^{-1}$ to -60°C and thawing from -60°C were 17% ± 7%. In cultures which had been plunged into LN, viability was 36% ± 4% post-thaw. These cells were observed to be larger than the largest of the untreated control cells [Fig. 5.4 (blue; i, j)]. A similar response was observed in cells exposed to potentially lethal osmotic stresses, *e.g.*, 4M NaCl, 2M NaCl and 1M NaCl solutions where 26% ± 2%, 30% ± 3% and 29% ± 0.3% respectively, of the cells were found to be larger than the untreated control cells [Fig. 5.4 (k-m)]. Only

after 48 h. of recovery did a proportion of cells (5% ± 3%) which had been lethally injured by a direct plunge into LN show an increase in size [Fig. 5.4 (yellow; f)].

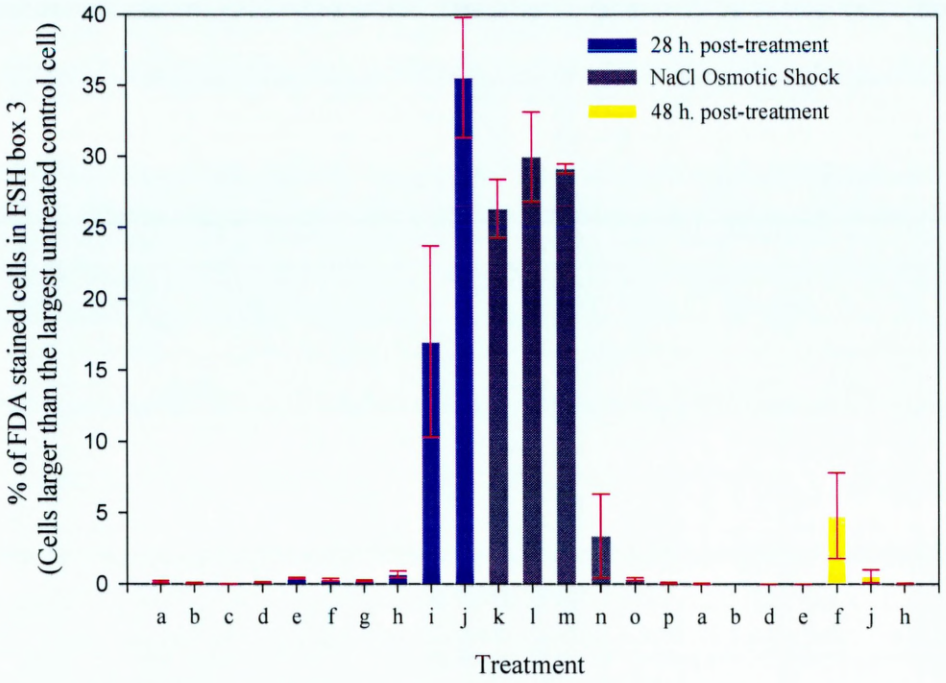


Figure 5.4 Effect of cooling rate and osmotic shock on the size of *Euglena gracilis* cells.

(a) Unstained untreated control, (b) FDA stained untreated control, (c) Cell centrifuged for 1 min., (d) Cells exposed to cryoprotectant at 0°C (10 % (v/v) methanol) for 15 min., (e) Unstained, cells plunged directly into LN, without cryoprotectant, (f) Cells plunged directly into LN, without cryoprotectant, (g) Cells control cooled at -0.5°C $min.^{-1}$ to -60°C and held for 30min., (h) Cells control cooled at -0.5°C $min.^{-1}$ to -60°C and held for 30min. then plunged into LN, (i) Cells control cooled at -0.3°C $min.^{-1}$ to -60°C and held for 30min., (j) Cells control cooled at -0.3°C $min.^{-1}$ to -60°C and held for 30min. them plunged into LN, (k) Cell exposed to 4M NaCl, (l) Cell exposed to 2M NaCl, (m) Cell exposed to 1M NaCl, (n) Cell exposed to 0.5M NaCl, (o) Cell exposed to 0.25M NaCl, (p) Cell exposed to 0M NaCl. All frozen samples were thawed using a simple two-step protocol.
n = 3, errors are expressed as standard errors of mean.

Further studies into cellular damage and recovery in *E. gracilis* focused on the effect of individual treatment steps of cryopreservation protocol and the recovery period on the cellular photosynthetic capacity (Fig. 5.5). The photosynthetic capacity of cells exposed to 0°C was reduced by 45% ± 1%, however, this did not prove to be lethal [Fig. 5.1 (b)]. Furthermore, exposure to the cryoprotectant at 0°C [methanol 10% (v/v)] reduced the photosynthetic oxygen evolving capacity by 46% ± 5% [Fig. 5.5 (d)]. However, on removal from the cryoprotectant the cells were subsequently able to recover their full

photosynthetic capacity [Fig. 5.5 (c)]. The duration of the recovery period was demonstrated to influence cellular photosynthetic capacity after exposure to -60°C, with photosynthetic oxygen evolution capacity increasing from 17% ± 3% after 24 h. recovery to 48% ± 8% after a further 24 h. [Fig. 5.5 (f, h)]. Little change in photosynthetic capacity was observed between 24 h. and 48 h. post-thaw in cells which had been exposed to LN. The cultures photosynthetic oxygen evolving capacity was 13% ± 2% and 16% ± 4%, of the untreated control, respectively (Fig. 5.5).

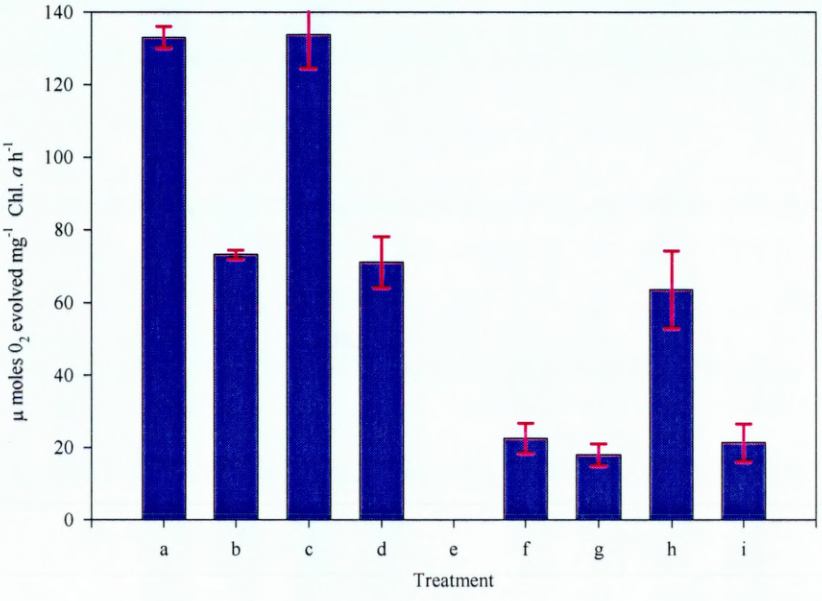


Figure 5.5 Photosynthetic activity of *Euglena gracilis* determined after exposure to different stages of a two-step controlled cooling protocol immediately after thawing and after 24 h. of post-thaw recovery.

(a) Untreated control cells, (b) Cells cooled to 0°C and held for 15 min., (c) Removed from cryoprotectant (10% (v/v) methanol) after 15 min. exposure at 0°C, (d) Cells exposed to cryoprotectant for 15min. at 0°C, (e) Cells plunged directly into LN, no cryoprotectant present, (f) Cells cooled from 0°C at -0.5°C $min.^{-1}$ to -60°C and held for 30min., time zero (g) Cells plunged into LN from -60°C, time zero. Cells were thawed using a simple two-step protocol. (h) Cells cooled from 0°C at -0.5°C $min.^{-1}$ to -60°C and held for 30min., 24 h. (i) Cells plunged into LN from -60°C, 24 h. Cells were thawed using a simple two-step protocol.
n = 3, errors are expressed as standard errors of mean.

5.3.2 *Haematococcus pluvialis*

A series of investigations were also performed on the unicellular chlorophyte *H. pluvialis*. Photosynthetic capacity in *H. pluvialis* was affected by exposure to the cryoprotectant [DMSO 5% (v/v)] (Table 4.3). In addition, exposure to DMSO 5% (v/v) was demonstrated to promote cellular division in aplanospore stage *H. pluvialis* (Table 3.6).

Table 5.1 Effects of different cryopreservation regimes on the photosynthetic capability and viability of *Haematococcus pluvialis*

Treatment[a]	Photosynthetic Capacity[b]	% Viability [c]
Control [d] (22°C)	100 ± 4.6	100 ± 0
Control [d] (0°C)	126 ± 3.2	95 ± 2
DMSO[e] (22°C)	69 ± 3.8	92 ± 2
DMSO[e] washed [f] (22°C)	74 ± 1.5	92 ± 2
0 h.[g] 22°C → -35°C [h,i]	137 ± 2.1	92 ± 3
0 h.[g] 22°C → -35 °C → -196 °C [h,i,j]	118 ± 15.5	94 ± 1
24 h.[k] 22°C → -35°C [h,i]	82 ± 4.3	92 ± 2
24 h.[k] 22°C → -35 °C → -196 °C [h,i,j]	91 ± 3.5	94 ± 1
-196°C direct [l]	0 ± 0	0 ± 0

[a] Treatment step.
[b] Expressed as % of untreated control.
[c] Expressed as % of FDA stained untreated control.
[d] Untreated control (temperature).
[e] Exposed to cryoprotectant 5% (v/v) DMSO for 14 min.
[f] Removed from cryoprotectant.
[g] Photosynthetic capability recorded immediately post-treatment.
[h] Filaments incubated for 15 min. in the presence of DMSO (5% v/v).
[i] Cooled at -1°C min.$^{-1}$ to -35°C
[j] Cooled at -100°C min.$^{-1}$ from -35°C
[k] Photosynthetic capability recorded 24 h. post-treatment.
[l] Plunged into LN, without cryoprotectant.

n = 3, errors are expressed as standard errors of mean.

5.3.3 *Vaucheria sessilis*

Exposure to the cryoprotectant [methanol 10% (v/v)] and cooling to 0°C influenced the photosynthetic capacity of *V. sessilis*. However, after a subsequent recovery period filaments were able to recover their full photosynthetic oxygen evolving capacity (Fig. 5.6). Immediately after treatment levels were reduced by 70% ± 10% when methanol was added at 0°C and by 90% ± 3% when it was added at room temperature. However, filaments subsequently recovered fully on removal from the cryoprotectant (Fig. 5.6). Where filaments were cooled to subzero temperatures, and the extracellular environment froze, an initial depression of the oxygen evolution rate of > 80% occurred. On subsequent incubation under standard culture conditions oxygen evolution rates returned to close to their original level (Fig. 5.6).

Cooling the filaments to the point when intracellular ice occurred, resulted in a total loss of photosynthetic oxygen evolvution, these filaments ultimately bleached and died. In all cases where filaments retained the capacity to evolve oxygen, they were able to grow and generate new filaments (Fig. 5.6, Chapter 3). No significant bleaching, or variation in chlorophyll concentration, was noted during the recovery of these filaments; [Time zero 680 ± 50 μg Chl. *a*. g^{-1} (wet weight); 24 h. post-treatment; 680 ± 60 μg Chl. *a*. g^{-1} (wet weight). 48 h. post-treatment; 630 ± 30 μg Chl. *a*. g^{-1} (wet weight)].

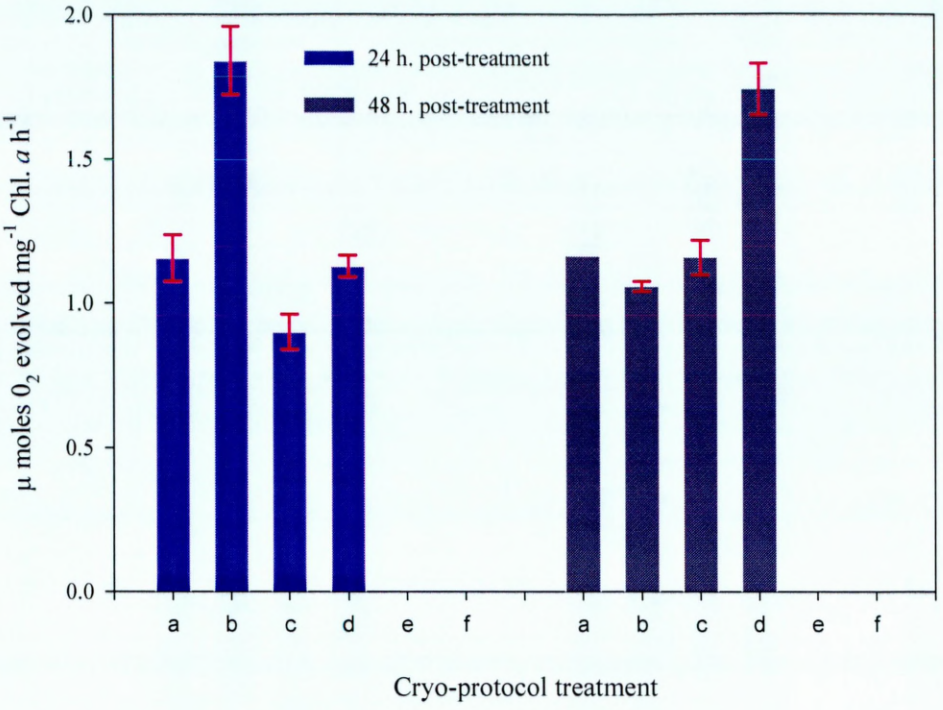


Figure 5.6 The effects of cryoprotectant and cooling on the oxygen evolution of *Vaucheria sessilis*. Recovery of oxygen evolving capacity 24 h. and 48 h. post treatment.

(a) Untreated control, (b) Filaments exposed to cryoprotectant (10% (w/v) methanol), (c) Filaments supercooled in cryoprotectant, (d) Filaments supercooled without cryoprotectant, (e) Filaments two-step cooled to -35 °C in cryoprotectant, (f) Filaments plunged into LN from -35°C in cryoprotectant.
n = 3, errors expressed as standard errors of mean.

5.4 Discussion

Investigations of cellular responses to chilling and freezing using vital staining and the measurement of algal photosynthetic capacity confirmed that the algae were not simply chill sensitive. This was demonstrated by the capability of all cells to recover from cooling to 0°C without loss of viability (Figs. 5.1, 5.6, Table 5.1). Furthermore, high post-thaw viability levels could be attained for all organisms exposed to subzero temperatures: *E. gracilis* and *V. sessilis* both survived cooling to -10°C with no reduction in viability and *H. pluvialis* could be recovered after exposure to LN with viability in excess of 80% (Chapter 3).

5.4.1 Investigation of cellular damage and recovery in *Euglena gracilis*

During two-step cryopreservation employing uncontrolled cooling rates, no viable cells were recovered from either the intermediate temperature -35°C or from LN (Fig. 3.1). This was attributed to insufficient dehydration of the cells due to the comparatively rapid initial cooling rate and the nucleation of ice within cells. In studies discussed in Chapter 4, where a range of controlled cooling rates were employed, all cells experienced intracellular ice formation during cooling at rates greater than -1°C $min.^{-1}$ to -60°C. This ice nucleation event was considered to be lethal (Fig. 4.1).

5.4.1.1 Rapid cooling > -1°C $min.^{-1}$

To further test the hypothesis of lethal injury at cooling rates > -1°C $min.^{-1}$ to -60°C, flow cytometry was employed to assess viability after cooling at faster than -1°C $min.^{-1}$ (Fig. 5.1). Vital staining with FDA indicated that all cells cooled at these rapid cooling rates were lethally damaged. At these cooling rates, lethal injury may be attributed to insufficient cellular dehydration, leading to intracellular ice formation (see 1.9.2-1.9.3). A successful two-step cryopreservation protocol would have enabled the concentration of the intracellular solution to reach a point at which it formed a glass, preventing any further concentration of the supercooled cytosol (Steponkus *et al.*, 1992). This would only occur if sufficient time was allowed for osmotic equilibrium to take place, permitting cryodehydration to progress to a point where the cytosol vitrifies in preference to homogeneous ice nucleation (Steponkus *et al.*, 1992). Provided, cells are not exposed to injurious solute concentrations, this phase change may confer protection from the potentially lethal frozen environment (Pegg, 1987).

5.4.1.2 Cooling at -1°C $min.^{-1}$

By employing a cooling rate of -1°C $min.^{-1}$ to -60°C, viable cells of *E. gracilis* could be recovered after exposure to -60°C but not after exposure to LN (Fig. 5.1). It is likely that at this slower cooling rate sufficient concentration of the cytosol had occurred to avoid lethal intracellular ice nucleation during cooling to the intermediate holding temperature

(-60°C). This was confirmed by the cryomicroscopic studies reported in Chapter 4, in which only a proportion of the cells cooled at -1°C min.$^{-1}$ were observed to undergo a "flashing" event, attributable to intracellular ice nucleation, during cooling to -60°C.

Further cooling by plunging cells directly into LN proved lethal for all cells. This could be attributed to one of two modes of injury. 1) Intracellular ice nucleation during the plunge into LN, as a result of insufficient cryodehydration of the cytosol, or 2) recrystallisation/fracture events due to instabilities in the intracellular space during thawing. Recrystallisation and fracture events should have been avoided through the adoption of a two-step thawing protocol which slowly warmed cells to -100°C, allowing vitreous material to soften, avoiding fractures, followed by rapidly warming cultures to ambient temperature avoiding ice crystal growth (Pegg *et al.*, 1997). It is therefore likely that lethal injury occurred due to an intracellular ice nucleation event during cooling. In addition, ice nuclei formed during cooling may grow during thawing further injuring the cell (Taylor, 1987). Intracellular "flashing" events were clearly observed during the cryomicroscopic investigations of Chapter 4, on cooling the alga to below -60°C, this further supports the hypothesis of lethal injury due to intracellular ice formation (Table 4.1).

5.4.1.3 Slow cooling < -1°C min.$^{-1}$

By employing still slower cooling rates of -0.5°C min.$^{-1}$ and -0.3°C min.$^{-1}$ to -60°C, sufficient cryodehydration of the cytosol should be possible to preclude intracellular ice nucleation events (Steponkus *et al.*, 1992). Vital staining of *E. gracilis* with FDA, assessed using flow cytometry, confirmed that viable cells could be recovered after exposure to both -60°C and LN (Fig. 5.1). However, neither cooling rate resulted in 100% post-thaw viability (Fig. 5.1). Cooling at -0.5°C min.$^{-1}$ to -60°C resulted in the highest post-thaw viability levels after exposure to both -60°C and LN, 68% ± 2% and 49% ± 4% respectively [Fig. 5.1 (magenta; f, g)].

Cells cooled at -0.3°C min.$^{-1}$ to -60°C had greatly reduced levels of viability after exposure to both -60°C and LN (21% ± 5% and 8% ± 2% respectively [Fig. 5.1 (light

blue; f, g)]). It was unlikely that this reduction in viability, could be attributed to intracellular ice nucleation, because "flashing" events were not observed using cryomicroscopy and on cooling at -0.5°C $min.^{-1}$ high levels of post-thaw viability were attained. It is therefore likely that lethal injury on cooling at -0.3°C $min.^{-1}$ was due to excessive cryodehydration causing a rise in solute concentration which was not fully mitigated by the cryoprotectant (Steponkus *et al.*, 1992; Pegg, 1987). During a series of investigations measuring changes in molarity in unfrozen water during the course of a cooling protocol, the molarity of the unfrozen proportion of the solution increased from 0.15 M to 5 M by -30°C in pure water, however, in water containing 3.0 M glycerol the molarity of the unfrozen solution reached a plateau at < 1 M (Morris, 1981).

Studies to determine the effect of osmotic shock on *E. gracilis* showed that the organism could tolerate NaCl concentrations as high as 0.5 M with little or no loss of viability (Fig. 5.3). However, on increasing the concentration to 1 M, viability was reduced to 34% ± 1% and higher concentrations resulted in 100% mortality (Fig 5.3). It is possible that the abrupt drop in viability observed between cells exposed to 0.5 M an 1 M NaCl may explain the significantly lower viability levels when cells were cooled at -0.3°C $min.^{-1}$ rather than at -0.5°C $min.^{-1}$. On cooling at -0.3°C $min.^{-1}$ lethal injury may be due to the cells being exposed to either an excessively long period of exposure and/or excessively high solute concentrations. It is possible that during cooling at -0.5°C $min.^{-1}$ the cells reached the maximum solute concentration they could tolerate and/or the maximum period which they could tolerate exposure to the elevated solute concentrations (Fig. 5.1). This, in turn, may suggest that the cells are being exposed to a high level of non-lethal stress due to the elevated solute concentration which may be exhibited as reversible free radical mediated injury. Free radical injury due to desiccation/dehydration has been reviewed by Benson (1990) and the principles of cryodehydration are discussed in 1.9.2-1.9.3. Further evidence for reversible injury due to the effects of dehydration were reported in studies on the effect of osmotic stress on *Chlamydomonas reinhardtii*, where following osmotic shrinkage and rehydration, fragmentation of the mitochondrial network was observed within potentially viable cells (Morris *et al.*,1985).

5.4.1.4 Optimum cooling rate (-0.5°C min.$^{-1}$)

After cooling at the optimum rate of -0.5°C min.$^{-1}$ and exposure to -60°C and LN, viability levels were reduced to 68% ± 2% and 49% ± 4% respectively. This suggests that 32% ± 2% of cells experienced lethal injury during the cryopreservation protocol. Lethal injury may be due to a series of events including: intracellular ice nucleation (5% of cells) during cooling to -60°C (Fig. 4.1) and natural senescence. In addition, the possibility of a proportion of cells experiencing excessive cryodehydration cannot be precluded. However, a proportion of lethally injured cells remain unaccounted for. This, together with the detection of false positive results, reported in Chapter 3 (up to 24 h. post-thaw), promotes the possibility that cells may be experiencing a series of stresses which may encourage free radical mediated injury. These stresses may ultimately culminate in lethal injury in a proportion of cells. The possibility of free radical injury, both lethal and non-lethal, is investigated in Chapter 7. Furthermore, to attain the high post-thaw viability levels reported, required the adoption of a two-step warming protocol, as discussed in Chapter 3.

The implementation of a preliminary slow thaw was likely to have permitted relaxation of the vitreous state, avoiding potentially lethal freeze fracture events permitting high post-thaw viability levels to be attained after exposure to LN (Fig. 5.2). Although, this may only be quantified with DSC considerable evidence exists in support of this hypothesis. Thawing via either a simple two-step protocol (vials were thawed by slow warming in the air for 1 min. followed by rapid warming in a pre-heated 40°C water bath), or by controlled two-step thawing (placing the vials in a programmable freezer pre-cooled to -130°C for 30min. followed by immersion in a pre-heated 40°C water bath), permitted high post-thaw viability levels to be attained (65% ± 0.5% and 64% ± 4% respectively) [Fig. 5.2 (g, h)]. When vials were thawed using a controlled thawing rate of +100°C min.$^{-1}$ to ambient temperature, viability was reduced to 26% ± 5% [Fig. 5.2 (i)] and the reduction in viability may be attributed to recrystallisation and/or ice crystal growth during thawing. However, at this warming rate relaxation of the vitreous environment would be likely, avoiding fracture events. When vials were thawed using a simple single-step direct immersion, post-thaw viability levels were reduced still further

to 5% ± 1% [Fig. 5.2 (j)]. The reduction in viability may be due to exposure to the elevated and therefore potentially damaging temperatures, within the water bath (40°C). However, a 40°C water bath was also employed during the simple two-step thawing protocol, this technique resulted in comparatively high viability levels, therefore it is unlikely to account for the very low levels of survival. It can therefore be hypothesised that the lethal injury may be due to freeze fracture events and/or recrystallisation (Pegg, 1997).

Changes in cell size were dependant upon the type of injury which cells had experienced. In cells which had been exposed to lethal osmotic injury, either through excessive cryodehydration or exposure to high molarity NaCl solutions, an increase in cell size was detected [Fig. 5.4 (i-m)]. This may be a result of the lethally injured cells loosing their ability to retain their normal elongate form and/or osmoregulate. However, because the injury had not disrupted the cells' pellicle structure enlarged cells resulted. When cells were lethally injured through intracellular ice nucleation, damage to the cell pellicle may occur and cause the cytoplasm to be lost through cell lysis and so preclude the formation of enlarged cells.

Cell lysis, modifications at the cell membrane level, break down of fine structures and/or loss of cellular compartmentalisation have all previously been proposed as potential mechanisms of freezing injury and are discussed in detail in 1.11-1.11.1.2. The flow cytometry data was recorded 24 h. and 48 h. post-thaw and this incubation period would have permitted severely damaged cells to die and damaged membranes to rupture. This may explain why cryomicroscopic studies, which only measured volume changes for the period immediately following thawing, failed to identify enlarged cells (Fig. 4.3). Cryomicroscopy did detect enlarged cells immediately post-thaw, following freezing at -10°C $min.^{-1}$ which may be due to lethal injury preventing the cells from retaining/regaining their elongate form, prior to membrane rupture/breakdown. The small number of enlarged cells detected 48 h. after thawing following direct immersion in LN < 5% (Fig. 5.4) may be due to the aggregation of grossly ruptured pellicle material, which the flow cytometer detected as a large, non-FDA stained event.

In addition to lethal/non-reversible injury during freezing, *E. gracilis* may be experiencing non-lethal/reversible stresses during the cryopreservation protocol (Fig. 5.5). Photosynthesis/respiration assays demonstrated that the photosynthetic capacity of *E. gracilis* could be reduced by exposure to low-temperatures and/or cryoprotectant exposure (Fig. 5.5). It is therefore likely that the cryopreservation protocol was affecting the photosynthetic ability of the cells and may indicate that the chloroplasts were damaged by the cryopreservation protocol (Fig. 5.5). Furthermore, in cells which had been exposed to -60°C and whose photosynthetic capacity was measured during recovery, photosynthetic capacity was seen to increase from 17% ± 3% 24 h. post-thaw, to 48% ± 8% 48 h. post-thaw (Fig. 5.5). This may be attributed to non-lethal injuries which inhibited photosynthetic capability during the first 24 h. of recovery, however, during the subsequent 24 h. the cells were able to recover and regain much of their photosynthetic capacity. If these results are considered as a measure of viability then 42% ± 8% of the cells may be considered to be viable. Flow cytometry assessment of viability employing vital staining indicated that 68% ± 2% of cells were viable. The difference between the two figures may be due to a degree of continued photosynthetic inhibition, or an over estimation of viability by flow cytometry due to "false positives". Flow cytometry studies have demonstrated that enzymatic activity may continue, even in lethally damaged cells, however, where the cells were non-viable, or were unable to recover from the stresses to which they had been exposed during the cryopreservation protocol, false FDA staining was only detected up to 24 h. after thawing (Chapter 3).

The detected inhibition of photosynthetic capacity through exposure to low temperatures and/or cryoprotectant solution indicates that there may be a possible biochemical mode of cryoinjury. In addition, there is a probability that cells may experience both lethal and non-lethal stresses during cryopreservation. Little change in photosynthetic capacity was detected between 24 h. and 48 h. post-thaw in cells which had been exposed to LN, their photosynthetic capacity was 13% ± 2% and 16% ± 4% of control levels respectively. This may be attributed to the cumulative injurious events, which cells exposed to LN have experienced, culminating in either survival or lethal injury. The cells which survived would be the most robust cells and may not experience prolonged photosynthetic inhibition. However, in cultures thawed from -60°C an additional group

of non-lethally injured cells would be present and these cells may experience prolonged photosynthetic inhibition. In addition, there is the possibility of inhibition of photosynthetic capacity beyond 48 h. of recovery in cells exposed to -60°C and LN.

The inhibition of photosynthesis in *E. gracilis* cells exposed to low and subzero temperatures may indicate that chloroplasts are a specific site of low temperature stress/injury. Chloroplast damage by exposure to low temperatures and freezing have been described (Ginsburger-Vogel *et al.*, 1992; Morris *et al.*, 1985; Heber *et al.*, 1971; 1981). Ultrastructural studies have shown a range of damage related symptoms including: swelling and distortion of the thylakoids, formation of small vesicles at the envelope, accumulation of lipid droplets and disintegration of the envelope (Benson, 1990; Ginsburger-Vogel *et al.*, 1992) [Figs. 4.10, 4.6 (B)]. In addition, to ultrastructural changes, freezing of thylakoid membranes may cause inactivation of phosphorylation (Heber *et al.*, 1971; Santarius, 1987; 1990a,b,c; 1996). Furthermore, cryoprotective compounds which protect intact cells against freezing also protect thylakoid membranes against inactivation of phosphorylation (Heber *et al.*, 1971). These reports indicate that the thylakoid membranes may be especially sensitive of chilling and freezing injury. Thylakoid membranes in TEM sections of chilling stressed *V. sessilis* were clearly disrupted and accumulation of lipid deposits was readily associated with these membranes (Fig. 4.10). The inactivation of the thylakoids by cryoprotectant exposure and low temperature may therefore induce a reduction in photosynthetic capacity immediately after treatment.

5.4.2 Investigations of cellular cryodamage and recovery in *Vaucheria sessilis*

Cryoprotectant toxicity and the period of exposure to increased salt concentrations may be significant causes of freezing injury and subsequent death (Pegg, 1987) and were categorised by Levitt (1980) as secondary freeze-induced injury and cryoprotectant toxicity, however, studies on the oxygen evolving capacity of *V. sessilis* demonstrated that low-temperature and exposure to cryoprotectant could induce the reversible inhibition of the oxygen evolving capacity in the alga. Exposure to the cryoprotectants employed initially reduced the photosynthetic capacity of *V. sessilis* by 70% ± 10%

when added at 0°C and by 90% ± 3% when added at room temperature. However, filaments subsequently recovered fully on removal from the cryoprotectant, as assessed 24 h. and 48 h. after treatment (Fig. 5.6). These events may be attributed to non-lethal stress in the alga. In addition, during examination of TEM sections, reported in Chapter 4, in material exposed to low temperatures (-10°C) a large accumulation of lipid droplets associated with chloroplast thylakoid membranes were observed. It is likely that an effect of chilling stress on *V. sessilis* was being manifested as the accumulation of lipid droplets in the chloroplasts (Fig. 4.10). Investigation of the photosynthetic capacity in cells cooled to -12°C demonstrated a > 80% reduction in photosynthetic capacity, however, after a suitable period of recovery photosynthetic capacity returned to close to 100% (Fig. 5.6). This may indicate that chilling was directly or indirectly inhibiting metabolic pathways. In addition, inactivation of the thylakoids by cryoprotectant exposure and low temperature could cause the reduction in oxygen evolving capacity immediately after treatment. Many of these changes can be induced in samples prior to exposure to the frozen environment, therefore secondary freeze induced injury (Levitt, 1980), may instead be due to a complex series of events including: oxidative stress, inhibition of metabolic pathways and the effects of cryodehydration. Each of these events may in turn contribute to the freeze recalcitrance in this alga.

Chloroplasts and in particular thylakoid membranes may be damaged by chilling and freezing (Benson, 1990; Heber *et al.*, 1971; 1981; Ginsburger-Vogel *et al.*, 1992). Ultrastructural studies have shown a range of damage related symptoms including: swelling and distortion of the thylakoids, formation of small vesicles at the envelope, accumulation of lipid droplets and disintegration of the envelope (Wise *et al.*, 1983). At 5°C symptoms were time dependant with the time course being related to chilling injury (Wise *et al.*, 1983). A comparable range of ultrastructural alterations were observed in *V. sessilis* (Fig. 4.10). Inactivation of thylakoid membranes by freezing has been described and is more extensive the higher the concentration of solute and has been described as differing from the kinetics of inactivation due to ice formation (Heber *et al.*, 1971; Santarius, 1987). In addition, chilling enhanced photooxidation, "bleaching", has been reported and may account for the rapid bleaching of mortally injured filaments (Wise, 1995) (Table 3.8).

Studies on cells which had been cooled to lower subzero temperatures resulted in no demonstrable photosynthetic capacity (Figs. 5.6, 3.9). In these filaments cryomicroscopic investigations had previously indicated that intracellular ice nucleation was likely (Table 4.3, 4.4). TEM sections also indicate that considerable damage to filament organelle integrity had occurred, particularly in the thylakoid arrangement (Fig. 4.10). This gross disruption by intracellular ice was a probable cause of the loss of photosynthetic capacity and ultimately filament mortality (Fig. 5.6, Table 4.3).

5.4.3 Investigation of cellular damage and recovery in *Haematococcus pluvialis*

H. pluvialis was found to be tolerant to the effects of cryopreservation (Chapter 3). Therefore this series of investigations did not attempt to determine lethal injury, instead post-thaw metabolic changes due to chilling and/or cryoprotectant exposure were investigated. Studies reported in Chapter 3 indicated that enhanced recovery could be observed in cells treated with DMSO [5% (v/v)]. This response was attributed to DMSO influencing the cells metabolic capacity and promoting cell division in aplanospore stage cells. The ability of DMSO to promoting cell division have previously been reported (Hahne & Hoffmann, 1984; Beauchamp & Crete, 1968; Herschler, 1968). These studies have indicated that exposure to DMSO [5% (v/v)] could initially inhibit photosynthetic capacity (69% ± 3.2%), which subsequently rapidly recovered on removal from the cryoprotectant (Table 5.1). In addition, enhanced photosynthetic activity was observed immediately post-warming (Table 5.1). This may be due to disruption/uncoupling of electron transport chains in the photosystems and "overshoot" responses in the chloroplasts caused by chilling and freezing injury. Previously Heber *et al.* (1971) reported that thylakoid membranes may be damaged by freezing and that inactivation of photophosphorylation may occur. Cold temperatures have been reported to slow the energy-consuming Calvin-Benson Cycle enzymes more than the energy-transducing light reactions, thus causing leakage of energy to oxygen and this has been linked to chilling enhanced photooxidation (Wise, 1995). In addition, after a freeze-thaw treatment of isolated spinach thylakoid membranes inactivation of photophosphorylation may occur (Santarius, 1987). This membrane damage has been reported to increase in H^+ permeability of the thylakoid membrane and stimulate linear

photosynthetic electron transport whilst simultaneously the uncoupled electron flow decreased, reflecting a partial inhibition of the electron transport chain (Santarius, 1987). These effects and "overshoot" responses when inhibited electron pathways are re-established may account for the elevated oxygen levels immediately post-thaw in *H. pluvialis*, which has been demonstrated to be amenable to cryopreservation (Chapter 3). The effect of chilling and freezing on the photosynthetic capacity of *H. pluvialis* may therefore represent a non-lethal, reversible injury/stress. However, after a subsequent 24 h. of recovery the photosynthetic capacity returns to the levels of untreated control cells and may in turn be employed as a measure of post-treatment viability (Table 5.1).

5.4.4 Conclusions

The techniques employed have proven effective in the investigation and localisation of cryoinjury in the algae studied. Furthermore, they proved to be both robust and readily adaptable. However, flow cytometry may be further developed through the application of more specific fluorescent probes (Haugland, 1996) to localising freeze induced events. In addition, rapid developments in computer technology combined with new fluorescent dyes and monoclonal antibody production will further increase the potential of flow cytometry as an application for the investigation of cryoinjury and stress (Drouet & Lees, 1993).

Throughout this study increasing evidence of both lethal and non-lethal injury, which may inhibit metabolic pathways within algae has been presented (see Figs. 5.5, 5.6). The possibility of cell damage being associated with free radical mediated injury has been emphasised in this Chapter and Chapter 4. Evidence of this include: the inhibition of photosynthetic capacity and ultrastructural changes due to chilling, freezing and cryoprotectant exposure. This may result in increased free radical activity within chloroplasts which, in turn, may overwhelm the cells antioxidant defence systems and result in free radical mediated injury. Free radical activity and antioxidant status are investigated and discussed in greater detail in Chapters 7 and 8.

Chapter 6.

Novel cryopreservation approaches.

Contents **Page No.**

6.1 Introduction

The two-step cryopreservation protocols developed/investigated in Chapters 3-5 commonly started with a period of exposure to a cryoprotectant solution (5-10% w/v) which: either a) concentrated the intracellular material through the removal of intracellular water by osmotic action of and/or b) replaced a proportion of the intracellular water with the cryoprotectant. A two-step cooling procedure was then employed, where the cells were cooled to a pre-selected intermediate temperature, prior to plunging in LN. Initially, two-step cooling was achieved by plunging cells into an immersion bath pre-cooled to an intermediate temperature (industrial methylated spirits (IMS) bath at -30°C) followed by a plunge into LN. This provided a comparatively rapid and uncontrolled initial cooling rate and was employed with some success (Chapter 3). The greatest successes were obtained using controlled cooling rates employing programmable coolers (Chapter 3, 5) (Day & McLellan, 1995a,b). Both approaches to two-step cooling exploit the fact that chilling injury is dependent upon the rate of cooling (Grout *et al.*, 1990).

In cryopreservation protocols which utilise moderate concentrations of cryoprotectants and cooling rates, where ice formation is inevitable, glass formation still occurs resulting in the "unfrozen" water fraction of these solutions vitrifying (MacFarlane, 1987). However, the formation of a vitreous state remains an intrinsic property of all liquids, pure water and aqueous solutions will readily vitrify given a sufficiently high cooling rate (MacFarlane, 1987) and this may be exploited for the development of novel cryopreservation protocols (Sakai *et al.*, 1991; Fabre & Dereuddre, 1990; Armitage, 1986). This is a potentially desirable attribute and may be employed in an attempt to circumvent ice formation altogether, resulting in the entire solution entering a glassy state (1.9.2-1.9.6) (MacFarlane, 1987; MacFarlane *et al.*, 1992; Franks, 1985). This may be achieved by employing highly concentrated solutions which readily vitrify (MacFarlane, 1987; MacFarlane *et al.*, 1992; Steponkus *et al.*, 1992; Mehl, 1996a,b). Cellular material may then be preserved through immobilisation in an amorphous solid. Alternatively, vitrification may be achieved through the desiccation of a tissue (concentrating the cytosol) (Dereuddre, 1992; Dumet *et al.*, 1993a,b) or desiccation of

encapsulated tissue followed by rapid cooling to -196°C (Dereuddre, 1991; Dereuddre *et al.*, 1992). The principles of vitrification have been discussed in detail in 1.9.2, 1.9.6.2.

6.1.1 Vitrification

Conventional vitrification aims to prevent intracellular ice growth, limit or exclude the exposure of the material to the dehydration effects of extracellular ice, extracellular injury due to freezing of the media, mechanical stresses, desiccation problems and remove the requirement for specialised expensive equipment (1.9.6.2) (Meryman & Williams). This is most commonly achieved by adopting rapid cooling rates and employing highly concentrated solutions (Sakai *et al.*, 1991; Armitage, 1989). However, these solutions are prone to problems due to their high osmotic potentials and potential toxicity (1.9.6.2) (Steponkus *et al.*, 1992). A characteristic vitrification solution may employ molecular liquids (*e.g.*, propylene glycol) at concentrations in excess of 40% (w/w) (MacFarlane, 1987). In attempts to reduce the toxicity of the vitrification solutions mixtures of cryoprotectants have been developed including sugars. Constituents of the vitrification solution may include glycerol, sucrose, ethylene glycol and DMSO (Sakai *et al.*, 1991; Yamada *et al.*, 1991; Nishizawa *et al.*, 1993). However, the toxicity of these highly concentrated vitrification solutions to the algae was demonstrated in toxicity studies performed to assess the toxicity of much less concentrated two-step cryopreservation solutions (Chapter 3). These studies indicated that *Euglena gracilis* was damaged by exposure to DMSO at concentration as low as 5% (v/v) and that duration of cryoprotectant exposure also influenced viability (Tables 3.3, 3.4). *Vaucheria sessilis* was also found to be sensitive to exposure to various cryoprotectants at concentrations of between 5% and 10% (v/v) (Table 3.7). The elevated concentrations of vitrification solutions, may result in mortality, this makes the application of vitrification using chemical additives to the algae problematic.

In addition, to the problems of toxicity, vitrified material may be prone to fracturing, this has been suggested as a basis of cellular mortality (Hunt *et al.*, 1994; Pegg *et al.*, 1997). The fracture events are believed to be initiated at points of ice nucleation and crystal growth which cause irreversible mechanical damage within the cell, this is discussed in detail in 1.9.2 and by Mehl (1996b). However, vitrification does present the

possibility of circumventing many of the injurious events which have been identified during two-step cryopreservation techniques, *e.g.*, intracellular ice nucleation, ice crushing and excessive cryodehydration (Chapters 3-5). It may be possible to employ alternative methodologies which may permit the vitrification of cellular material without the requirement for highly concentrated/toxic vitrification solutions. The exposure to both highly concentrated vitrification solutions or the dehydration of encapsulated cells will allow concentration of the cytosol to a degree at which its T_h (the homogeneous nucleation temperature) is sufficiently low to permit cooling to below its thermodynamic freezing point, through its metastable supercooled regime (avoiding both homogeneous and heterogeneous ice nucleation), and finally below its "glass transition" temperature T_g forming a vitreous material (Stillinger, 1995). Encapsulation/dehydration may therefore present a mode of employing vitrification to cells which are particularly sensitive to cryoprotectant toxicity.

6.1.2 Encapsulation/dehydration

Encapsulation has been employed in a number of disciplines for many diverse applications, of which a few have been discussed in 1.9.6.3. In addition, the encapsulation of cells in sodium alginate for cryopreservation was developed by Fabre and Dereuddre (1990) and many examples have been detailed in 1.9.6.3. Furthermore, encapsulated algal cells are more readily manipulated during cryopreservation protocols and subsequent recovery procedures. In cryobiology, the majority of current applications of encapsulation involve the dehydration of the encapsulated cells/alginate followed by a rapid plunge into LN in an attempt to achieve a vitrified state (Dereuddre *et al.*, 1992; Bachiri *et al.*, 1995; Gonzalez-benito *et al.*, 1997). In encapsulated somatic embryos dehydrated and subsequently rapidly cooled by plunging in LN, differential scanning calorimetry studies have demonstrated that protection may be conferred via a vitrification process (Dereuddre *et al.*, 1992). However for encapsulation/dehydration to be successful cells must survive the dehydration process and achieve sufficient concentration of the cytosol to a degree at which its T_h (the homogeneous nucleation temperature) is sufficiently low to permit cooling of the cytosol below its thermodynamic freezing point, avoiding both homogeneous and heterogeneous ice nucleation, to below T_g forming a vitreous material (Stillinger, 1995). This may prove

difficult for those planktonic microalgae which are not commonly exposed to dehydration in their natural aquatic environment. However, there are many algae and microalgae which do experience extremes of dehydration and high osmotic stress in their natural environment (Bold & Wynne, 1985) which may be expected to survive vitrification by encapsulation/dehydration (1.5.2, 1.5.5). The application of desiccation to the preservation in higher plants has been discussed in 1.7.6.4. These newly developed techniques, have employed direct desiccation of somatic embryos with considerable success (Villalobos & Engelmann, 1995; Uragami *et al.*, 1992; Dereuddre, 1992; Uragami *et al.*, 1993; Dumet *et al.*, 1993a,b; Janeiro *et al.*, 1996). Although the majority of the microalgae adopted for investigation are more commonly found in aquatic environments, encapsulation of dehydration intolerant cells may allow the principles of dehydration/vitrification to be applied to these algae. *Haematococcus* spp. may be maintained as aplanospores for extended periods in a sealed dry containers (Leeson *et al.*, 1984) (1.7.2.1-1.7.2.2). It was anticipated that these cells should survive a dehydration/vitrification preservation protocol.

6.1.3 Encapsulation/two-step cooling

A further adaptation of encapsulation methodology is the two-step controlled cooling of encapsulated material. This may limit the effects of exposure to the frozen crystalline environment, in addition, frozen materials of relatively low solubility, *e.g.*, alginate, have been reported to exhibit inhibition of spherulite ice crystal formation (Franks, 1985; Luyet & Rapatz, 1958) (1.9.2).The benefits of encapsulation have been applied to the two-step preservation of gametophytes of *Laminaria* (Vigneron *et al.*, 1997) (1.9.6.3). In addition, encapsulation has been employed as a procedure to avoid fracture damage during freezing and thawing of rabbit embryos (Kojima *et al.*, 1990). Studies comparing encapsulated and non-encapsulated embryos have reported that encapsulated embryos, cooled by conventional controlled two-step cooling techniques, have enhanced function and survival after thawing, which ultimately permits increased numbers of offspring from frozen and thawed embryos (Kojima *et al.*, 1990). An alternative to the controlled cooling of encapsulated cells involves the dehydration of encapsulated cells with a highly concentrated vitrification solution (PVS2) followed by rapid cooling by plunging in LN (Phunchindawan *et al.*, 1997).

6.1.4 Objectives

This Chapter aims to investigate the possibility of applying novel cryopreservation approaches to the preservation of the algae. Novel techniques for investigation included: vitrification in the presence of high molarity vitrification solutions, encapsulation/dehydration in sodium alginate and the controlled two-step cooling of encapsulated cells. Techniques were evaluated for their suitability for algal preservation and, where successful, the protocols protective mechanisms investigated. Successful preservation procedures, were developed to investigate the effects of the preservation treatment on the metabolic capacity of the algae and the applicability of the technique for algal long term preservation.

6.2 Materials and Methods

6.2.1 Organisms and culture regimes

Cultures of *Microcystis aeruginosa* Kützing emend. Elenkin CCAP 1450/8, *Anabaena cylindrica* Lemmermann CCAP 1403/2B, *Haematococcus pluvialis* Flotow CCAP 34/8, *Enteromorpha intestinalis* (L.) Link CCAP 320/1, *Euglena gracilis* Klebs CCAP 1224/5Z and *Vaucheria sessilis* (Vaucher) De Candolle ex Collins CCAP 745/1C were prepared for study. Full details on these strains are listed in 2.1. Culture regimes and recovery conditions were as described in 2.2-2.3. Filaments of the xanthophytic alga *Vaucheria sessilis* were prepared as described in 4.2.1.

6.2.2 Vitrification protocols

The suitability of employing vitrification to preserve algae was examined using two vitrification solutions developed by Sakai (Sakai *et al.*, 1991; Nishizawa *et al.*, 1993) which have been successful in permitting the preservation of higher plant species (Ishikawa *et al.*, 1997; Maruyama *et al.*, 1997; Matsumoto *et al.*, 1997; Matsumoto *et al.*, 1995a,b). Cells were vitrified using the sugar based vitrification solutions PVS2 (Yamada *et al.*, 1991) and PVS3 (Nishizawa *et al.*, 1993) prepared in appropriate media

(2.2). In addition, a number of hybrid recipes (based on PVS2 and PVS3) and alternative pre-culture regimes were tested. These additional mixtures are listed in the discussion (Table 6.9).

All vitrification solutions were prepared fresh and autoclaved at 10 bar for 15 min. Vitrification solutions were pre-cooled to 0°C and added to pre-cooled cell suspensions at 0°C. Cells were exposed to the vitrification solution at 0°C for either a 5 or 15 min., prior to being directly immersed in LN.

6.2.2.1 PVS2 solution

Cells were incubated in PVS2 solution, a mixture of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose prepared in the appropriate media (pH 5.8) (2.2) at 0°C for 5 or 15 min. prior to a plunge into LN (Yamada *et al.*, 1991).

6.2.2.2 PVS3 solution

Cells were treated using a two-step vitrification procedure. Initially cells were incubated in a mixture of 2 M glycerol and 0.4 M sucrose at either 0°C or 25°C for 10 min. The cells were then removed from this solution (by centrifugation) and dehydrated at 0°C for 5 or 15 min. prior to a plunge into LN with PVS3 solution containing 50% (w/v) glycerol and 50% (w/v) sucrose in water (Nishizawa *et al.*, 1993).

6.2.3 Encapsulation/dehydration protocols

Cells were encapsulated in sodium alginate at 2% (w/v) sodium alginate augmented with 0.5M sucrose (Hirata *et al.*, 1995); in 3% (w/v) sodium alginate (Benson, 1994); in 3% (w/v) sodium alginate augmented with 0.5M sucrose or in 5% (w/v) sodium alginate augmented with 0.5M sucrose.

Alginate solutions were prepared in the appropriate calcium free media (EG:JM or JM) with Alginic acid (sodium salt), Sigma A-0682 (Sigma, USA) and autoclaved for 15

min. at 10 bar. Media was prepared as detailed in 2.2, employing modified media recipes (Tables 6.1-6.3).

Table 6.1 Euglena gracilis medium for encapsulation solutions (EG)

EG (calcium free) was prepared by the addition of Sodium acetate trihydrate (1.0 g), "Lab-Lemco" powder (Oxoid L29) (1.0 g), Tryptone (Oxoid L42) (2.0 g) and Yeast extract (Oxoid L21) (2.0 g) to deionised water (per litre) (Tompkins *et al.*, 1995).

Table 6.2 Jaworski's medium for encapsulation solutions (JM)

Stock No.	Compounds	per 200 ml
1)	K_2HPO_4	2.48 g
2)	$MgSO_4.7H_2O$	10.0 g
3)	$NaHCO_3$	3.18 g
4)	EDTA (FeNa Salt)	0.45 g
	EDTA (Na_2 Salt)	0.45 g
5)	H_3BO_3	0.496 g
	$MnCl_2.4H_2O$	0.278 g
	$(Na_4)_6Mo_7O_{24}.4H_2O$	0.20 g
6)	Cyanocobalamin	0.008 g
	Thiamine HCl	0.008 g
	Biotin	0.008 g
7)	$NaNO_3$	16.0 g
8)*	$Na_2HPO_4.12\ H_2O$	7.2 g

* stored at room temperature.
JM was prepared by the addition of 1.0 ml of each stock solution to deionised water (per litre) (Tompkins *et al.*, 1995).

Table 6.3 EG:JM medium for encapsulation solutions

This medium was prepared using a 1:1 mixture of EG and JM. (Tables 6.1, 6.2) Media were mixed and then autoclaved (Tompkins *et al.*, 1995).

Cultures were centrifuged and the supernatant removed prior to encapsulation. The concentrated cell suspension was then mixed with an appropriate amount of alginate (1ml cell suspension to 20ml alginate) and dispensed drop wise, using an Eppendorf multipette 4780 (Eppendorf, Germany), into 100mM $CaCl_2$ solution prepared in dH_2O. The encapsulated cells were incubated in the $CaCl_2$ solution for 15 min., the beads were then by filtering them through a sterile mesh sieve. The beads were then lightly dried on sterile filter paper and transferred to the appropriate pre-culture media. Encapsulated cells were pre-cultured for 24 h. in 0.5M sucrose supplemented media, followed by a further 24 h. in 0.75M sucrose supplemented media. Alternatively encapsulated cells were dehydrated for 48 h. in 0.75M sucrose supplemented media.

Beads containing encapsulated cells were then removed from the pre-culture media by filtering through a sterile mesh sieve, lightly dried on sterile filter paper and transferred to a laminar flow cabinet in the base of a sterile, Petri dish. Encapsulated cells were air dried in a laminar flow cabinet with forward flowing air flow for 1 h. - 6 h. Dehydrated beads were then plunged directly into LN.

6.2.4 Encapsulation/two-step cooling protocols

Alginate solutions were prepared as described in 6.2.3. Cell suspensions were mixed with alginate and dispensed as detailed in 6.2.3. The cryoprotectant solutions employed were prepared as described in 4.2.2. Vials were cooled to their intermediate holding temperature (-60°C at -0.5°C $min.^{-1}$) using a Planer Kryo 10 programmable freezer (Planer, UK) and held at this temperature for a 30 min., prior to being plunged directly into LN.

6.2.5 Thawing

All vials were thawed using a two-step protocol. This initially allowed vials to slowly warm, while being held in the air, for 1 min. followed by rapid warming in a pre-heated 40°C water bath.

All vials were agitated in the water bath until the last ice crystals had melted (Day *et al.*, 1997). The thawed material was then aseptically transferred to appropriate fresh, sterile, medium (2.2), and used in the viability assays as outlined in 2.5. All errors are expressed as standard errors of mean.

6.2.6 Recovery from the cryostore after long-term cryostorage

Vials which were maintained frozen in long-term investigations were transferred from the CCAP cryostore to a small Dewar containing liquid nitrogen (2.4) and transferred to the laboratory for thawing (6.2). Viability was assessed measuring chlorophyll *a* levels after 7 days recovery under standard conditions in a fixed volume of media (3 ml) (2.2, 2.5.6, 2.5.6.2).

6.2.7 Scanning electron microscopy

6.2.7.1 SEM of *Euglena gracilis*

Preparation of *E. gracilis* cells for scanning electron microscopy (SEM) followed a specific fixing protocol to avoid flagellar, loss as described in 4.2.5.1. Cells were maintained in suspension at all times preventing flagellum loss, fixatives and alcohol steps were as described in 2.6.3. SEM was performed on critical point dried *E. gracilis* cells coated with gold as described in 2.6.3.1.

6.2.7.2 SEM of encapsulated *Euglena gracilis*

Encapsulated cells were fixed at room temperature for 4 h. in buffered (0.1M sodium cacodylate buffer) glutaraldehyde [4% (w/v)] followed by a 30 min. buffer wash. They

were further fixed for 2 h. in buffered osmium tetroxide [2% (w/v)], followed by two further 30 min. buffer washes and two 30 min. deionised water washes.

Fixed encapsulated cells were then dehydrated in a graded ethanol series to 100% ethanol and in 100% ethanol overnight as detailed in 2.6.3. SEM was performed on critical point dried *E. gracilis* cells coated with gold as described in 2.6.3.1.

6.2.8 Flow cytometry

Flow cytometry was carried out on cells after a predetermined recovery period of either 24 h. or 48 h. using a FACStar Plus flow cytometer (Becton Dickinson, UK) as described in 2.5.8, using the vital stain fluorescein diacetate (FDA) prepared in methanol (2.5.2.1).

6.2.9 Chlorophyll and oxygen electrode measurements

6.2.9.1 Chlorophyll extraction and measurement

A known wet weight of sample was heated, in the dark, at 80°C in 3 ml methanol for 20 min., chlorophyll levels were then determined as described by MacKinney (1941) (2.5.6, 2.5.6.2).

6.2.9.2 Photosynthetic capacity

Respiration and oxygen evolution rates were obtained using a Rank oxygen electrode according to Whitelam and Codd (1983). Illumination was constant and non-limiting for oxygen evolution steps (440 μmol m^2 s^{-1}) (2.5.7). Beads were suspended above the electrode (sensor) and magnetic "flea" on a platinum mesh (5.1.2). This arrangement reduced noise due to bead movement, whilst permitting efficient circulation of the medium ensuring maximum sensitivity.

6.3 Results

6.3.1 Vitrification

Vitrification was employed as a technique for the preservation of the algae studied, however, only *Enteromorpha intestinalis* was capable of tolerating both exposure to the vitrification solution, and vitrification, with high levels of post-exposure viability (100%) (Table 6.4). *Euglena gracilis* was unable to tolerate exposure to the vitrification solutions employed (Table 6.4). *Microcystis aeruginosa*, *Haematococcus pluvialis* and *Vaucheria sessilis* were able to tolerate 5 min. exposure to PVS2, however, no viable cells (as measured by generation of new cells) were recovered after exposure to LN or after prolonged exposure to the cryoprotectant at 0°C (15 min.) (Table 6.4).

Table 6.4 Effects of vitrification solutions and vitrification on the post-exposure survival of selected strains of algae

Organism	PVS2 [a]	PVS3 [b]	PVS2/LN [c]	PVS2/LN [d]
E. gracilis	-	-	-	-
H. pluvialis	+	N/D	-	N/D
V. sessilis	+	-	±	-
M. aeruginosa	+	N/D	-	N/D
E. intestinalis	+	N/D	+	N/D

[a] Exposed to PVS2 at 0°C for 5 min.
[b] Exposed to PVS3 at 0°C for 5 min.
[c] Exposed to PVS2 at 0°C for 5 min. or 15 min. then plunged into LN.
[d] Exposed to PVS3 at 0°C for 5 min. or 15 min. then plunged into LN.

+ Positive post-treatment viability
- No viable cells post-treatment
± Filaments retained their colouration and appeared to undergo limited growth prior to death.
N/D Not determined

n = 3

6.3.1.1 Vitrification of *Euglena gracilis*

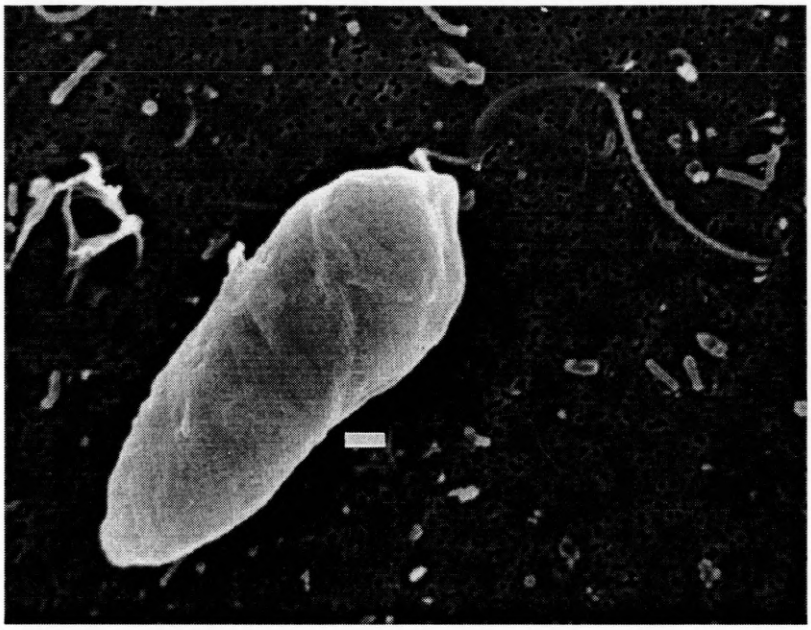


Figure 6.1 SEM micrograph of *Euglena gracilis* showing the cell with an attached flagellum.
Scale bar represents 1μm.

Viable cells of *E. gracilis* (Fig. 6.1) were not be recovered after exposure to any of the vitrification solution employed (Table 6.4). Therefore, it was to be expected that no viable cells would be recovered after exposure to LN (Table 6.4).

6.3.1.2 Vitrification of *Vaucheria sessilis*

V. sessilis filaments vitrified in PVS2 solution, after pre-culture in JM medium supplemented with 0.09M sucrose, retained their green colouration in a small proportion of filaments for over 1 month post-thaw. However, no measurable filament growth was observed and all filaments ultimately bleached and died (Table 6.4). *V. sessilis* filaments were able to tolerate up to 15 min. exposure to the vitrification solution at 0°C. However, exposure at 22°C resulted in > 75% mortality (determined by filament bleaching) within 4 days.

6.3.1.3 Vitrification of *Haematococcus pluvialis*

Although *H. pluvialis* was able to tolerate exposure to the vitrification (PVS2) solution for up to 5 min., no viable cells were recovered after exposure to LN, or after prolonged incubation in the vitrification solution (PVS2) at 0°C (15 min.) (Table 6.4).

6.3.1.4 Vitrification of *Enteromorpha intestinalis*

Enteromorpha intestinalis was successfully vitrified using a PVS2 vitrification solution (Table 6.4). Fracturing of the vitreous solution was observed during thawing which "shattered" the *E. intestinalis* filament leaving the thawed material with a macerated appearance. However, all recovered filament sections were ultimately able to generate new filaments of *E. intestinalis*. However, the recovery period between thawing and visible filament regeneration/growth could be a long as 4 weeks.

6.3.2 Encapsulation/dehydration

Algal cells were encapsulated in sodium alginate at concentrations of 2% (w/v) supplemented with 0.5M sucrose, 3% (w/v) supplemented with 0.5M sucrose, 5% (w/v) supplemented with 0.5M sucrose and 3% (w/v) without supplements. *E. gracilis*, *H. pluvialis*, *A. cylindrica* and *M. aeruginosa* were all successfully preserved and survived exposure to LN (Table 6.5). However, viability levels post-LN exposure appeared low for *H. pluvialis* and *M. aeruginosa*. In all cases where viable cells are encapsulated the cells were able to "grow" out of the alginate bead. The motile *E. gracilis* could be observed with microscopy to be "working its way out" of the alginate bead.

Table 6.5 Effects of encapsulation/dehydration on the post-thaw survival of selected strains

Organism	2% + 0.5M[a]	3% + 0.5M[b]	3%[c]	5% + 0.5M [d]
E. gracilis	-	-	-	+
H. pluvialis	+	N/D	N/D	N/D
V. sessilis	±	N/D	N/D	N/D
M. aeruginosa	+	N/D	N/D	N/D
A. cylindrica	+	N/D	N/D	N/D

[a] Thawed after exposure to LN, [encapsulated in 2% (w/v) sodium alginate supplemented with 0.5M sucrose].
[b] Thawed after exposure to LN, [encapsulated in 3% (w/v) sodium alginate supplemented with 0.5M sucrose].
[c] Thawed after exposure to LN, [encapsulated in 3% (w/v) sodium alginate].
[d] Thawed after exposure to LN, [encapsulated in 5% (w/v) sodium alginate supplemented with 0.5M sucrose].
+ Positive post-treatment viability
- No viable cells post-treatment
± Filaments retained their colouration and appeared to undergo limited growth prior to death.
N/D Not determined

n = 3

6.3.2.1 Encapsulation/dehydration of *Euglena gracilis*

E. gracilis was encapsulated and air dried prior to plunging into LN. Cells encapsulated in 3% (w/v) alginate supplemented with 0.5M sucrose experienced a reduction in weight due to dehydration during the first 3 h. of drying. Bead weight was then reduced to 29% ± 1% of the untreated control bead weight, further drying indicated that the bead had effectively reached a plateau and that further water loss due to dehydration was not occurring at a readily measurable rate (4 h. air drying reduced the bead weight to 27% ± 0.5%) (Fig. 6.2). When cells were encapsulated in 5% (w/v) alginate supplemented with 0.5M sucrose, weight loss due to dehydration effectively reached a plateau after 3 h. of air drying (47% ± 1%). Little change in bead weight was detected following subsequent

drying (Fig. 6.2). The beads % moisture content after 4 h. air drying was 50% ± 1.3% and after 5 h. was 59% ± 0.5%.

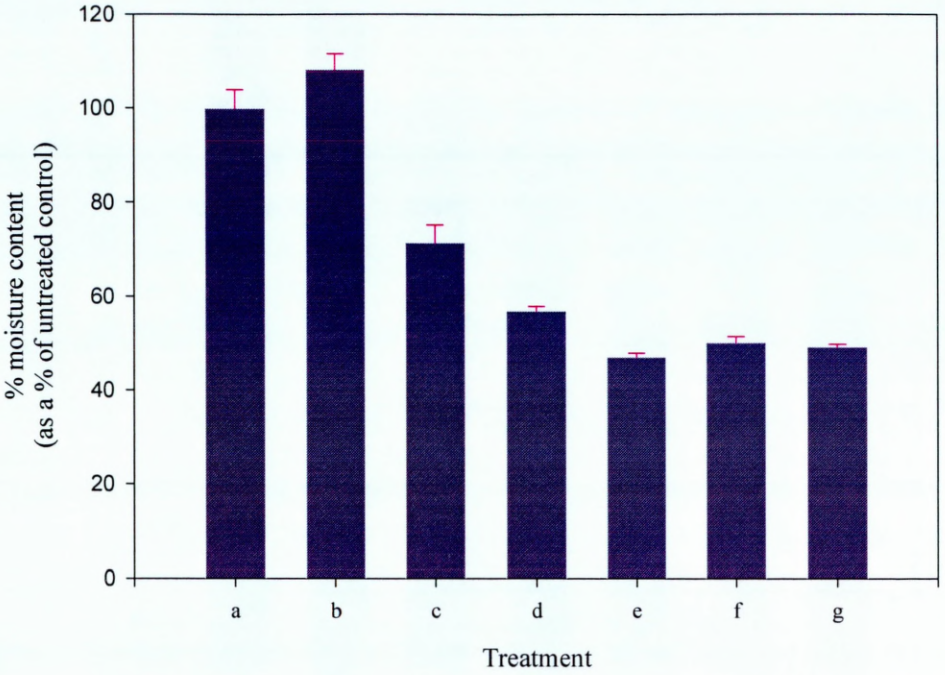


Figure 6.2 Change in encapsulated *Euglena gracilis* bead weight during 4 h. of air drying.

(a) Untreated control beads (3% alginate + 0.5M Sucrose) prior to 48 h. in 0.75M Sucrose (b) % moisture content of bead at start of air drying, time zero, (c) % moisture content after 1 h. air drying, (d) % moisture content after 2 h. air drying, (e) % moisture content after 3 h. air drying, (f) % moisture content after 4 h. air drying, (g) % moisture content after 5 h. air drying.

n = 3, errors are expressed as standard errors of mean.

Encapsulated *E. gracilis* cells were able to tolerate air drying and dehydration to varying degrees, depending upon the initial concentration of alginate. Although cells were able to tolerate air drying for up to 5 h. when encapsulated in 2% (w/v) alginate supplemented with 0.5M sucrose, no viable cells could be recovered after exposure to LN (Tables 6.5, 6.6). When encapsulated in 3% (w/v) alginate supplemented with 0.5M sucrose, cells were able to tolerate up to 3 h. of air drying with minimal loss of viability. However, on further air drying of 4 h. and 5 h. viability dropped to 10% ± 7% and 0% respectively (Table 6.6). It was interesting to note that this apparent limit, beyond which a significant reduction in viability was detected corresponded to the point where

reduction in bead weight due to dehydration began to plateau (Fig. 6.2). *E. gracilis* encapsulated in 5% (w/v) alginate supplemented with 0.5M sucrose also appeared to tolerate up to 3 h. of air drying with minimal loss of viability, however, viability levels reduced when dehydration was continued beyond this point (Table 6.6).

When *E. gracilis* cells encapsulated in 5% (w/v) alginate supplemented with 0.5M sucrose were recovered after exposure to LN, viable cells could be attained with 1 h.-5 h. of air drying (Table 6.6). However, optimum viability levels were achieved after 3 h. of air drying (37% ± 8%). Further air drying reduced post-thaw viability levels to 7% ± 0.1% (Table 6.6).

Exposure to the $CaCl_2$ solution could have potentially influenced *E. gracilis* viability levels. To assess this, liquid cultures of *E. gracilis* were exposed to 50mM and 100mM concentrations of $CaCl_2$ for fixed duration's between 1 min. and 30 min. and their viability, post-exposure, assessed using FDA staining and flow cytometry. Viability levels did not appear to be significantly affected by exposure to either 50mM or 100mM $CaCl_2$ solutions (Fig. 6.3).

Table 6.6 Effects of encapsulation/dehydration on the post-thaw survival of *Euglena gracilis* assessed as chlorophyll *a* level after 7 days recovery

Treatment	% Viability		
	2% + 0.5M[a]	3% + 0.5M[b]	5% + 0.5M[c]
Untreated Control	100	100	100
O/N 0.5M Sucrose	+	84 ± 1	119
O/N 0.75M Sucrose	+	98 ± 3	148
2 × O/N 0.75M Sucrose	+	N/D	69 ± 2
0 h. air drying	+	98 ± 3	69 ± 2
1 h. air drying	+	78 ± 9	178 ± 4
2 h. air drying	+	73 ± 1	181 ± 21
3 h. air drying	+	96 ± 3	183 ± 1
4 h. air drying	+	10 ± 7	75 ± 12
5 h. air drying	+	0 ± 0	75 ± 32
0 h. air drying → LN	-	0 ± 0	6 ± 0.1
1 h. air drying → LN	-	0 ± 0	25 ± 1
2 h. air drying → LN	-	0 ± 0	22 ± 2
3 h. air drying → LN	-	0 ± 0	37 ± 8
4 h. air drying → LN	-	0 ± 0	7 ± 0.1
5 h. air drying → LN	-	0 ± 0	7 ± 0.1

[a] Thawed after exposure to LN, encapsulated in 2% (w/v) sodium alginate supplemented with 0.5M sucrose.
[b] Thawed after exposure to LN, encapsulated in 3% (w/v) sodium alginate supplemented with 0.5M sucrose.
[c] Thawed after exposure to LN, encapsulated in 3% (w/v) sodium alginate.
[d] Thawed after exposure to LN, encapsulated in 5% (w/v) sodium alginate supplemented with 0.5M sucrose.

+ Positive post-treatment viability
- No viable cells post-treatment
± Filaments retained their coloration and appeared to undergo limited growth prior to death.
N/D Not determined

n = 3, errors are expressed as standard errors of mean.

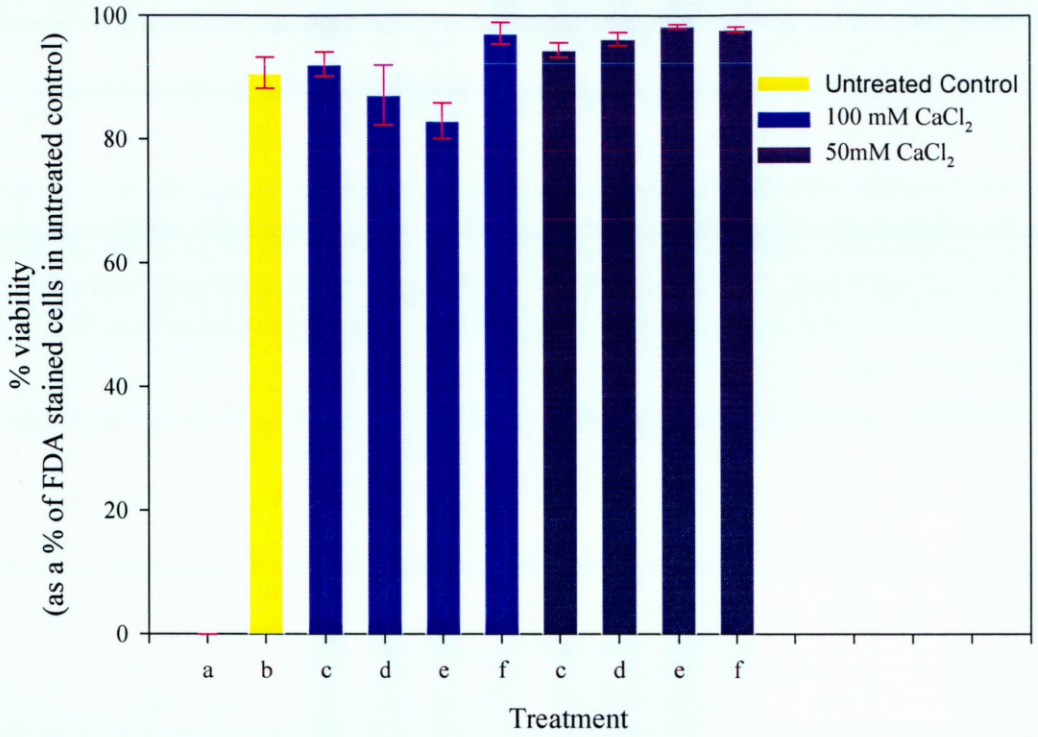


Figure 6.3 Effect of exposure period and concentration of $CaCl_2$ (50 and 100mM $CaCl_2$) on the post exposure viability of *Euglena gracilis*.

(a) unstained untreated control cells, (b) FDA stained untreated control cells, (c) 1 min. exposure, (d) 5 min. exposure, (e) 10 min. exposure, (d) 30 min. exposure.

n = 3, errors are expressed as standard errors of mean

6.3.2.2 Encapsulation/dehydration of *Vaucheria sessilis*

Encapsulated *V. sessilis* filaments were recovered after up to 5 h. of air drying. Material recovered after exposure to LN was lethally injured when they had been air dried for 1 h., 2 h. or 5 h. prior to being plunged into LN. Encapsulated filament which had been air dried for 3 h. or 4 h. retained their colour for two weeks following thawing. However, although it appeared some filament growth had occurred the recovered material was overgrown by fungal and bacterial contaminants (Table 6.5). Attempts were made to render this culture axenic, to allow these experiments to be repeated on non-contaminated *V. sessilis*. However, attempts to render the culture axenic were unsuccessful.

6.3.2.3 Encapsulation/dehydration of *Haematococcus pluvialis*

High viability levels were detected in *H. pluvialis* encapsulated in 2% (w/v) sodium alginate supplemented with 0.5M sucrose on up to 3 h. of air drying. Viable cells were recovered after exposure to LN after 2 and 3 h. of air drying (Table 6.5).

6.3.2.4 Encapsulation/dehydration of *Microcystis aeruginosa* and *Anabaena cylindrica*

Both *M. aeruginosa* and *A. cylindrica* were successfully preserved by encapsulation in 2% (w/v) sodium alginate supplemented with 0.5M sucrose after 3 h. of air drying in a laminar flow cabinet (Table 6.5).

6.3.3 Encapsulation/two-step cooling of *Euglena gracilis*

Preservation of *E. gracilis* by first encapsulating the cells, then controlled two-step cooling was found to be an effective and robust preservation protocol (Table 6.7). The highest post-thaw viability levels (> 40%) were attained when cells were encapsulated in 5% (w/v) alginate supplemented with 0.5M sucrose and either exposed to a graded pre-culture (24 h. in 0.5M sucrose and 24 h. in 0.75M sucrose), or a single step pre-culture (48 h. in 0.75M sucrose) (Table 6.7). To assess where an initial period of air drying may enhance post-LN exposure viability levels studies were performed on encapsulated cells which were air dried for fixed duration's of between 0 h. and 3 h. prior to cryoprotectant exposure (Table 6.8). No improvement in levels of post-treatment viability were achieved for encapsulated cells, which had been exposed to LN, and had an air drying step preceding the two-step control cooling (Tables 6.7, 6.8). In all cases viable cells were able to "swim out" of the alginate beads.

SEM studies of *E. gracilis* cells (Fig. 6.1) indicated that the alginate beads were effectively spherical, promoted uniform dehydration of the beads during air drying and even permeation of cryoprotectants (Fig. 6.4). Encapsulated *E. gracilis* cells were also investigated using SEM after exposure to key steps in the encapsulation/two-step control cooled protocol (Figs. 6.5-6.7).

Table 6.7 Effects of encapsulation/two-step cooling on the post-thaw survival of *Euglena gracilis* assessed as chlorophyll *a* level after 7 days recovery

Treatment	% Viability	
	3% + 0.5M[a]	5% + 0.5M[b]
Untreated Control	100	100
24 h. 0.5M Sucrose	84 ± 1	119 ± 3
24 h. 0.5M & 24 h. 0.75M Sucrose	98 ± 3	148 ± 4
48 h. 0.75M Sucrose	N/D	69 ± 2
10% (v/v) Methanol[c]	85 ± 3	128 ± 1.4
-0.5°C min^{-1} → -60°C[c]	27 ± 2	35 ± 2
-0.5°C min^{-1} → -60°C → LN[c]	30 ± 2	48 ± 1.5
10% (v/v) Methanol[d]	N/D	67 ± 0.2
-0.5°C min^{-1} → -60°C[d]	N/D	47 ± 0.6
-0.5°C min^{-1} → -60°C → LN[d]	N/D	44 ± 4

[a] Thawed after exposure to LN, [encapsulated in 3% (w/v) sodium alginate supplemented with 0.5M sucrose].
[b] Thawed after exposure to LN, [encapsulated in 5% (w/v) sodium alginate supplemented with 0.5M sucrose].
[c] Encapsulated cells pre-cultured for 24 h. in 0.5M sucrose and 24 h. in 0.75M sucrose.
[d] Encapsulated cells pre-cultured for 48 h. in 0.75M sucrose.
N/D Not determined

n = 3 errors are expressed as standard errors of mean.

Table 6.8 Effects of encapsulation/dehydration/two-step cooling on the post-thaw survival of *Euglena gracilis*

Treatment	% Viability	
	Untreated control [a]	0.75M Sucrose exposure [b]
Untreated Control	100	
48 h. in 0.75M Sucrose	82 ± 4	100
0 h. air drying [c]	95 ± 5	116 ± 6
1 h. air drying [c]	82 ± 8	101 ± 10
2 h. air drying [c]	64 ± 3	78 ± 3
3 h. air drying [c]	41 ± 10	50 ± 10
0 h. air drying → -60°C [d]	39 ± 1	47 ± 1
1 h. air drying → -60°C [d]	30 ± 1	37 ± 1
2 h. air drying → -60°C [d]	26 ± 1	32 ± 2
3 h. air drying → -60°C [d]	7 ± 0.1	8 ± 0.1
0 h. air drying → -60°C → LN [e]	20 ± 0.1	24 ± 0.1
1 h. air drying → -60°C → LN [e]	17 ± 0.2	21 ± 0.3
2 h. air drying → -60°C → LN [e]	20 ± 3	24 ± 4
3 h. air drying → -60°C → LN [e]	6 ± 1	8 ± 1

[a] Viability as a % of untreated encapsulated cells [5% (w/v) sodium alginate supplemented with 0.5M sucrose].
[b] Viability as a % of encapsulated cells post-48 h. incubation in 0.75M sucrose [5% (w/v) sodium alginate supplemented with 0.5M sucrose].
[c] Viability assessed after exposure to air drying in a forward venting laminar flow hood.
[d] Viability assessed after exposure to air drying in a forward venting laminar flow hood then cooled at -0.5°C $min.^{-1}$ to -60°C.
[e] Viability assessed after exposure to air drying in a forward venting laminar flow hood then cooled at -0.5°C $min.^{-1}$ to -60°C followed by a plunge into LN .

n = 3, errors are expressed as standard errors of mean.

Studies demonstrated that the alginate may act as a supporting structure around the cells with cells retaining their elongate form throughout the cryopreservation protocol (Figs. 6.6, 6.7). The cells appeared to have a two-layered alginate coating, the first of which was closely associated with the cells outer pellicle (Fig. 6.5). Beyond this was the main alginate coating which encased the cell within the bead (Fig. 6.5).

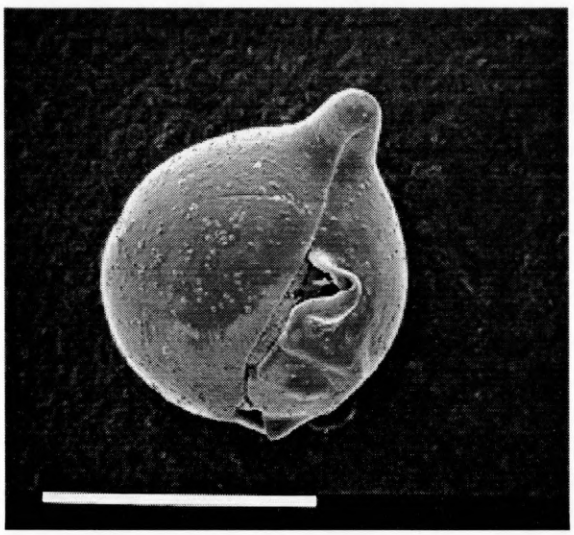


Figure 6.4 *Euglena gracilis* cells encapsulated in 5% (w/v) sodium alginate supplemented with 0.5M sucrose.
Scale bar = 1000μm.

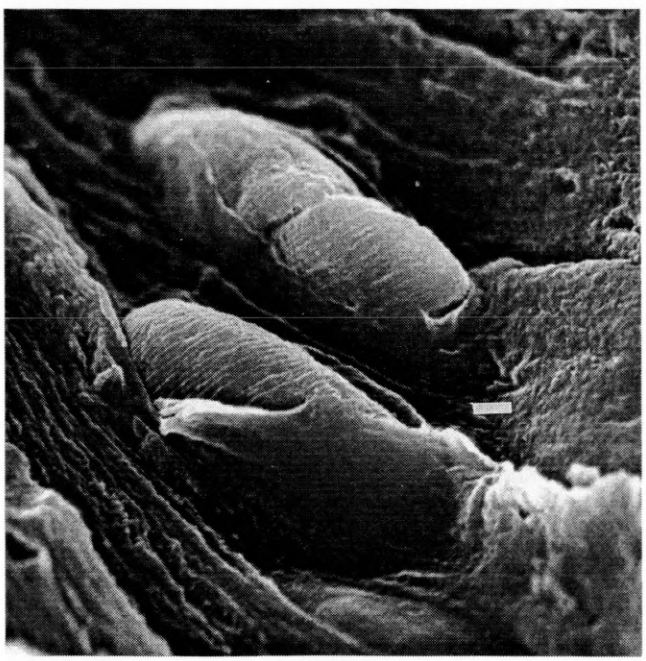


Figure 6.5 SEM of untreated *Euglena gracilis* cells embedded in 5% (w/v) sodium alginate supplemented with 0.5M sucrose.
Scale bar equals 1 μm.

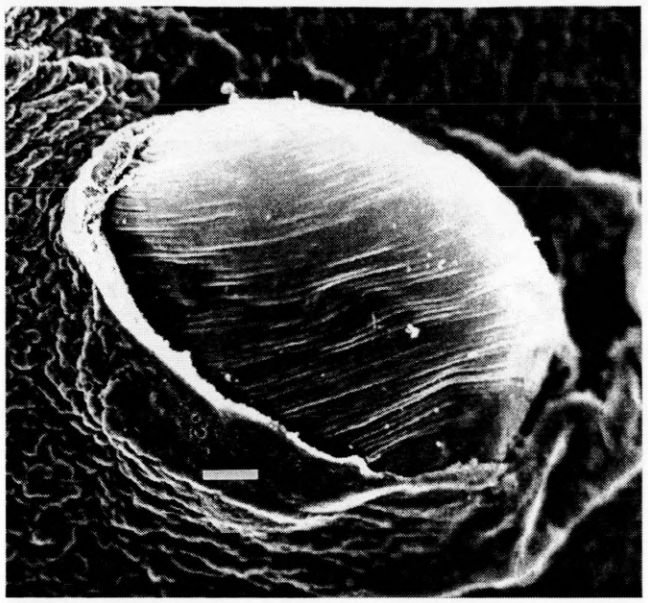


Figure 6.6 SEM of a *Euglena gracilis* cell embedded in 5% (w/v) sodium alginate supplemented with 0.5M sucrose, after controlled cooling to -60°C.
Scale bar equals 1 μm.

Figure 6.7 SEM of a *Euglena gracilis* cell embedded in 5% (w/v) sodium alginate supplemented with 0.5M sucrose after controlled cooling to -60°C and plunging into LN.
Scale bar equals 1 μm.

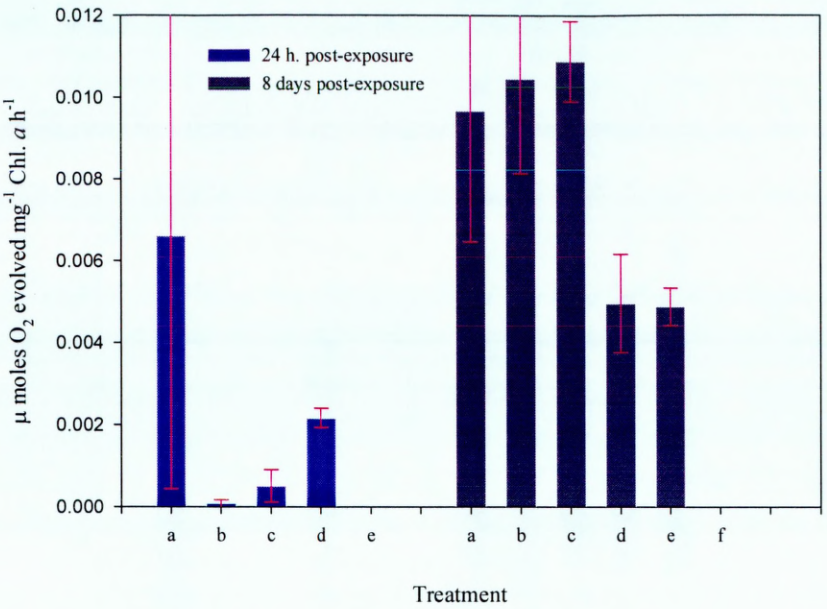


Figure 6.8 Photosynthetic capacity of encapsulated [5% (w/v) sodium alginate supplemented with 0.5M sucrose] *Euglena gracilis*, determined immediately after exposure to different steps of an encapsulation/two-step cooling protocol, 24 h. post-exposure and 8 days post-exposure.

(a) Untreated encapsulated control cells, (b) Encapsulated cells incubated for 48 h. in 0.75M sucrose EG:JM solution, (c) Encapsulated cells exposed to cryoprotectant [10% (v/v) methanol] for 15min. at 0°C, (d) Encapsulated cells cooled from 0°C at -0.5°C $min.^{-1}$ to -60°C and held for 30min., (e) Cells plunged into LN from -60°C, (f) Encapsulated cells plunged directly into LN, without cryoprotectant. All cells were thawed using a simple two-step protocol.

n = 3, errors are expressed as standard errors of mean.

After 8 days of recovery, post-exposure viability levels as assessed by the photosynthetic capacity of three beads for 48 h. incubation in 0.75M sucrose and 15 min. cryoprotectant [10% methanol (v/v)] at 0°C were 108% ± 24% and 113% ± 10% respectively of untreated encapsulated beads. The photosynthetic capacity after 8 days of recovery under standard conditions for encapsulated cells, recovered after exposure to -60°C and LN, were 51% ± 12% and 50% ± 5% respectively of the encapsulated untreated control cells (Fig. 6.8).

6.3.4 Long-term storage of encapsulated *Euglena gracilis*

Long term storage trials were implemented to assess the stability of encapsulated cultures after prolonged storage under LN. *E. gracilis* was preserved using an

encapsulation/two-step controlled cooling protocol in 5% (w/v) sodium alginate EG:JM solution supplemented with 0.5M sucrose. Beads were cooled at -0.5°C min.-1 to -60°C and held for 30 min. prior to being plunged into LN. Cells recovered using a standard two-step thawing procedures gave > 40% post-thaw viability 6 and 12 months after initial freezing.

6.4 Discussion

The application of novel approaches to the cryopreservation of algal cells has permitted viable cells to be recovered after exposure to LN. However, although a degree of success was achieved, a number of areas of cryoinjury specific to these novel techniques were identified.

6.4.1 Vitrification and encapsulation/dehydration

Vitrification solutions developed for use in plant and animal systems are commonly comprised of high concentrations of conventional cryoprotectants and/or more novel sugar constituents (Sakai *et al.*, 1991; De Paz *et al.*, 1994; Ishikawa *et al.*, 1997; Ishikawa *et al.*, 1996; Maruyama *et al.*, 1997). Although *E. intestinalis* was successfully vitrified the other algae examined failed to generate new cells after exposure to LN. In many cases the lethal injury was attributed to the toxicity of the vitrification solutions employed (Table 6.4). In particular *E. gracilis* proved unable to tolerate, even short-term exposure to vitrification solutions containing DMSO.

Many further hybrid vitrification solutions and vitrification procedures were assessed by the author for the cryopreservation of *E. gracilis* and *V. sessilis* (Table 6.9). None of these combinations of constituents or pre-culture regimes resulted in viable cells being recovered following exposure to LN (Table 6.9). In all cases where DMSO was employed as a constituent of the vitrification solution, lethal injury was attributed to the toxicity of the vitrification solution (Table 6.9). Furthermore, in less toxic vitrification solutions lethal injury was attributed to recrystallisation and/or freeze fracture events occurring in the vitrified material during thawing. In addition, no detectable benefit was achieved by the adoption of pre-culture regimes (Table 6.9).

Table 6.9 Alternative vitrification solutions and pre-vitrification strategies evaluated for *Euglena gracilis* and *Vaucheria sessilis* without successful recovery after exposure to LN

In addition to direct single step vitrification procedures algae were pre-cultured for 24 h. in a number of supplemented media recipes.

Pre-culture media recipes

- pre-culture in/on appropriate media supplemented with 0.09M sucrose
- pre-culture in/on appropriate media supplemented with 0.5M sucrose
- pre-culture in/on appropriate media supplemented with 1.2M sorbitol

Hybrid vitrification solutions

- 6% (v/v) DMSO
- PVS2 modified with 0.8M sucrose instead of 0.4M sucrose
- PVS2 modified with reduced/no DMSO with higher concentrations of other constituents
- 45% (v/v) DMSO supplemented with 20% (v/v) bovine serum albumin (BSA) and 1mg/l polyethylene glycol (PEG) (average molecular weight 8000) [Sigma, P-2139 (Sigma, USA)].
- 20% (v/v) DMSO supplemented with 30% (v/v) (BSA), 30% (w/v) sucrose and 1mg/l PEG.
- Encapsulated cells (6.2.3) were pre-cultured in 0.75M sucrose then rapidly dehydrated using PVS2 solution and vitrified in the presence of PVS2 (Phunchindawan *et al.*, 1997)

In addition, the author also employed encapsulation/vitrification protocol of Phunchindawan *et al.* (1997) to *E. gracilis*. Encapsulated cells were rapidly dehydrated using PVS2 solution and vitrified in its presence (Phunchindawan *et al.*, 1997). However, exposure to the vitrification solution (5 min.), even whilst encapsulated, proved lethal to the alga (Table 6.9, see also 6.4.2).

Monitoring of temperature fluctuations in the CCAP cryostore, indicated that there should be no significant temperature fluctuations during storage (Fig. 3.11). Assuming there were no deviations from the routine addition of liquid nitrogen, the thermal behaviour of the cryostore should be very stable over time. However, cells for cryopreservation in service culture collections, require to be maintained for extended periods and it is possible that cell damage may occur during routine manipulations (see Chapter 3). In vitrified samples or frozen samples with small ice crystals, recrystallisation or devitrification could potentially result in cellular damage. At liquid nitrogen temperatures the very small cubic ice (I_c) particles are thermodynamically metastable, due to their high surface energies and although crystal growth to larger, more stable hexagonal ice forms (I_h) generally occurs at high subzero temperatures, recrystallisation has been detected as low as -130°C and by X-ray diffraction techniques as low as -149°C (MacFarlane *et al.,* 1992; Taylor, 1987) (1.7.3). Studies performed to monitor temperature fluctuations during routine manipulation of the CCAP cryostore which simulated the removal/addition of vials stored at the bottom, middle and top of the inventory system demonstrated that fluctuations in temperature may be experienced both by the manipulated vial and vials in other sections of the inventory (Fig. 3.14). Furthermore, removal of a vial from the bottom of the inventory promoted a detectable rise in temperature to -112°C. At this elevated temperature there is the potential for changes in viability levels in cryopreserved material to be induced by devitrification, ice crystal growth and freeze-fracture events (Mehl, 1996a,b; Franks, 1985). Although the duration of exposure to these elevated temperatures was comparatively short cumulative injury in vitrified material retained for extended periods can not be precluded. In addition, once in the glassy state, the vitrified liquid's quantum states are frozen, however, the glassy state will relax towards its equilibrium state with time, dependant upon temperature, with the possibility of ice nucleation below the glass transition state (Mehl, 1996b). Vitrification may therefore have wider applications in the field of medical tissue preservation, where the period between preservation and tissue use is comparatively short in comparison to the periods of preservation proposed in microbiological culture collections. The application of vitrification techniques which first involve the immobilisation of cells in alginate and two-step cryopreservation of cells present the possibility of a more stable vitreous state which may be less susceptible

to temperature fluctuations than the more conventional high molarity vitrification solutions. The improved stability is due to the application of slower initial cooling rates, which correspond to a less stressed glass, furthermore the alginate itself also confers protection from freeze fracture events (Kojima, 1990). Scottez *et al.* (1992) also reported that the resistance of encapsulated/dehydrated shoot-tips to liquid nitrogen was not dependant on cooling rate.

6.4.2 Encapsulation/dehydration

Encapsulation/dehydration was successfully employed for the preservation of *H. pluvialis*, *A. cylindrica* and *M. aeruginosa*, however, viability levels post-LN exposure were much lower than those obtained using conventional two-step cooling (Table 6.5). Cell mortality appeared to be largely attributable to the dehydration step, with levels of viable cells being similar in dehydrated and LN exposed beads. Unfortunately the encapsulation of the cells made accurate assessment of viability difficult. In the detailed investigations performed using *E. gracilis*, viability was assessed by measuring the chlorophyll *a* content of three beads after a fixed recovery period under standard recovery conditions. The successful preservation of cells by encapsulation/dehydration requires that the cells be dehydrated to a point at which the cytosol is sufficiently concentrated to permit cooling to below its thermodynamic freezing point, without homogeneous ice nucleation, *i.e.*, to below its T_g thus forming a vitreous material (Stillinger, 1995). Although encapsulation in either 3% or 5% (w/v) sodium alginate supplemented with 0.5M sucrose for up to 3 h. of dehydration resulted in no significant loss of viability (96% ± 3% and 183% ± 21% respectively), only cells encapsulated in 5% (w/v) sodium alginate supplemented with 0.5M sucrose recovered after exposure to LN. It is possible that cells encapsulated in a 3% (w/v) alginate solution encountered lethal injury due to devitrification/fracture events, which were promoted by the lower alginate concentration/viscosity. These events were previously linked with lethal injury in *E. gracilis* in Chapter 5 (5.2). The most successful recovery of encapsulated cells was achieved when cells were encapsulated in a 5% (w/v) alginate solution (Table 6.6). However, the duration of dehydration influenced post-LN exposure viability levels. Beads which were insufficiently dehydrated would have been expected to encounter ice nucleation events during freezing and/or thawing. This was observed where

encapsulated cells were directly plunged into LN without a preliminary period of dehydration (viability was reduced to 6% ± 0.1% for untreated encapsulated cells). Beads which were dehydrated for 1 h. or 2 h. prior to cryopreservation, on thawing retained 25% ± 1% and 22% ± 2% respectively of their cells viable. Optimum viability was achieved when beads were dehydrated for 3 h. (37% ± 8%), this dehydration period corresponded with the point at which reduction in bead weight due to evaporation of water reached a plateau. Further dehydration reduced post-thaw viability levels (7% ± 0.1%) and this was attributed to cumulative injury due to the stresses associated with excessive dehydration. *E. gracilis* has previously been identified as being susceptible to injuries due to dehydration and osmotic stress in Chapter 5 (Figs. 5.1, 5.3).

It is clear that for encapsulation/dehydration to be a successfully employed in cryopreservation protocols, it is necessary for preserved material to have a high level of dehydration tolerance. In comparable studies on higher plant cells encapsulation/dehydration it has been reported that the level of dehydration tolerance may be enhanced using sodium alginate supplemented with sucrose and pre-cultured in a mixture of sucrose and glycerol (Matsumoto & Sakai, 1995; Niino & Sakai, 1992a,b; Niino *et al.*, 1992). The revised procedure may be applied to expand the applicability encapsulation/dehydration to less dehydration resistant materials (Matsumoto & Sakai, 1995; Niino *et al.*, 1992). Lilly meristems were found to yield optimum viability levels when they were encapsulated in alginate beads supplemented with 0.3M sucrose and then pre-cultured in a mixture of 0.8M sucrose plus 1M glycerol for 16 h. before dehydration (Matsumoto & Sakai, 1995). Pre-culture in media supplemented with sucrose prior dehydration, has also been reported to be effective in achieving successful cryopreservation of Ribes germplasm and apices by encapsulation/dehydration (Reed & Yu, 1995; Scottez *et al.*, 1992; Gonzalez-Arnao *et al.*, 1996). Although, many of the alginate solutions employed in this study were supplemented with sucrose and all encapsulation/dehydration protocols involved a predetermined duration of pre-culture in media supplemented with sucrose, viable cells could be recovered on up to 5 h. of air drying. However, only a 5% (w/v) alginate solution permitted viable cells to recovered after exposure to LN. In addition, a 5% (w/v) alginate solution supplemented with 0.5M sucrose permitted a longer period of dehydration than identically treated cells encapsulated in 3% (w/v) alginate solution supplemented with 0.5M sucrose (after 4 h.

air drying viability were 75% ± 12% and 10% ± 7% respectively) (Table 6.6). The alginate may confrere dehydration tolerance by trapping water and/or slowing the rate of dehydration. The more concentrated 5% (w/v) encapsulation solution may also provide greater support for the *E. gracilis* cells than the 3% (w/v) solutions.

Further adaptations to the encapsulation/dehydration technique have included the pre-culture of cells in media supplemented with 0.3M sucrose at 20°C prior to encapsulation in sodium alginate supplemented with a mixture of 2M glycerol plus 0.4M sucrose (Matsumoto *et al.*, 1995a,b). In these studies encapsulated meristems were dehydrated with highly concentrated vitrification solutions (PVS2 or PVS3) for about 100 min. at 0°C prior to being plunged into liquid nitrogen (Matsumoto *et al.*, 1995a,b). Although this technique has proven successful when applied to some higher plant species, when a similar technique was applied to *E. gracilis* 100% cell mortality occurred after only 5 min. exposure to the vitrification solution. The vitrification solutions, PVS2 and PVS3 both contain comparatively high concentrations of DMSO which has been reported to be extremely toxic to *E. gracilis* in Chapter 3 (Table 3.3).

The encapsulation process, dropping cells mixed with the sodium alginate into $CaCl_2$ solution at either 50mM or 100mM does not appear to be a source of lethal cellular injury (Fig. 6.3). Although the exposure step may induce non-lethal/reversible stress/injury, the 48 h. period between encapsulation and exposure to dehydration and/or LN, makes it unlikely that $CaCl_2$ exposure influenced post-treatment viability levels.

6.4.3 Encapsulation/two-step cooling

Encapsulation/two-step controlled cooling in 10% (v/v) methanol was employed for the successful preservation of *E. gracilis* (Table 6.7). The SEM micrographs of encapsulated *E. gracilis* cells have revealed that the alginate may act as a supporting structure around the cells. Cells observed using SEM, which had been fixed immediately post-treatment appeared to have retained their elongate form throughout the cryopreservation protocol (Figs. 6.6, 6.7). The cells appeared to have a two-layered alginate coating, the first of which was closely associated with the cell's outer pellicle (Fig. 6.5). This first alginate coating may be due to gelation of the alginate, by linking of

alginate molecules by cations, through exposure to Ca^{2+} ions in the EG:JM media surrounding the cells (held in place by surface tension). Beyond this is the main alginate coating which encases the cells within the bead and was formed by dropping the alginate/cell mixture into a solution of Ca^{2+} ions (Fig. 6.5). The direct support of the encapsulated cells by the alginate, may protect the cells from flagellar loss, which has previously been suggested as a possible site of pellicle weakness (Chapters 3, 4). In addition the alginate may act as an outer synthetic "membrane" reducing the rate and "shock" of osmotic change due to cryoprotectant exposure and/or cryodehydration.

Encapsulation/two-step cooling has been reported to permit high levels of viable gametophytes of the alga *Laminaria digitata* to be recovered post-LN (Vigneron *et al.*, 1997) (1.7.6.3). Pre-culture in sucrose supplemented media and partial dehydration of the gametophytes prior to cooling was reported to maximise levels of viable cells post-thaw (Vigneron *et al.*, 1997). Encapsulation/two-step cooling of *E. gracilis* was not improved by introducing a partial dehydration step (Table 6.8). Instead, lower viability levels were attained after LN exposure (Fig. 6.8), this was attributed to the *E. gracilis* being a particularly dehydration sensitive organism.

The encapsulation of cells prior to two-step controlled cooling may also provide protection from direct exposure to the frozen crystalline environment and alginate, has been reported to inhibition of spherulite ice crystal formation (Luyet & Rapatz, 1958) (1.7.3). In addition, encapsulation may reduce fracture damage during freezing and thawing (Kojima *et al.*, 1990). In studies comparing encapsulated and non-encapsulated embryos it has been reported that encapsulated embryos cooled by conventional controlled two-step cooling techniques have enhanced function and survival after thawing, ultimately permitting increased numbers of offspring from frozen and thawed embryos (Kojima *et al.*, 1990).

Further protection during both encapsulation/dehydration and encapsulation/two-step cooling protocols may be due to the affinity that sodium alginate has to metals, including the transition metals, (Cu, Zn, and Cd) (Crist *et al.*, 1994a,b). Soluble ionised salts of alkaline earth and heavy metals may bind with alginate, precipitating it from solution (*i.e.*, the formation of a gelatinous or flocculant precipitate, the bead), and

alginate has been reported to form salts with: sodium, potassium, ammonium, magnesium, triethanolamine, calcium, aluminium, zinc, copper, chromium, iron (ferrous), iron (ferric) and silver (McDowell, 1974). This ability to bind metal ions may permit alginates to influence free radical production by removing metal ion catalysts which are involved in many free radical production pathways (1.11-1.12). Freeze-thaw treatments have previously been linked to the production of damaging free radicals (Santarius, 1987; Heber *et al.*, 1971; Wise, 1995). The oxidative stress promoted by low temperature induces the possibility of highly toxic hydroxyl radicals being produced via the Fenton and the Haber-Weiss reaction pathways, with the Fenton reaction being catalysed by transition metal ions (1.11.2). The ability of alginate to bind transition metal ions may prevent/reduce the production of toxic hydroxyl radicals and so reduce radical mediated injury in freeze/thaw exposed *E. gracilis* cells (1.11.2). An alternative approach attempt to inhibit injury in chilled and frozen systems (mammalian transport organs and cryopreserved rice cells) by introducing free radical scavengers and exogenous compounds which chelate Fe^{3+} ions has previously been reported (Benson *et al.*, 1995; Fuller & Green 1986; Green *et al.*, 1986b; Healing *et al.*, 1989) (1.11.9).

The effects of each stage of the cryopreservation protocol on the alga was difficult to investigate whilst the cells were encapsulated. However, it was relatively straight forward to monitor oxygen evolution of the entrapped cells (5.1.2). Photosynthetic studies performed 24 h. post-thaw were subject to considerable "noise", and photosynthetic capacity may well have been inhibited by the treatments to which the alga had been exposed (Fig. 6.8). This reduction in oxygen evolution rates was also observed in cell suspension studies (Fig. 5.5). However, the studies carried out in Chapter 5 indicated that when the cells had regained their photosynthetic capacity, the oxygen electrode could be reliably employed to assay viability. By using this technique viability levels for encapsulated *E. gracilis* cells after exposure to the cryoprotectant [methanol 10% (v/v)], controlled cooling at -0.5°C $min.^{-1}$ to -60°C and plunging into LN from -60°C were determined. Oxygen evolution rates and hence viability levels were determined after 8 days of recovery. Following 48 h. incubation in 0.75M sucrose and 15 min. exposure to cryoprotectant [10% methanol (v/v)] at 0°C photosynthetic oxygen evolving capacity was 108% ± 24% (for cells exposed to the sucrose incubation step) and 113% ± 10% (for cells incubated in sucrose and exposed to cryoprotectant) of

untreated encapsulated cells. The photosynthetic capacity after 8 days of recovery under standard conditions for encapsulated cells, recovered after exposure to -60°C and LN, were 51% ± 12% and 50% ± 5% respectively of the encapsulated untreated control cells (Fig. 6.5-6.7).

Long-term storage trials were performed on *E. gracilis* cells which had been encapsulated in 5% (w/v) alginate solution supplemented with 0.5M sucrose and two-step cooled at -0.5°C $min.^{-1}$ to -60°C and plunged into LN after a 30 min. holding period at -60°C. Cells were recovered, after 6 and 12 months storage in the CCAP cryostore, using a standard two-step thawing procedure and cultured under standard conditions for 7 days. Viability levels at the initiation of the long-term study and after both 6 and 12 month storage were > 40%. This demonstrates that cryopreservation by encapsulation/two-step cooling has the potential to be employed for the long-term storage of genetic resources.

6.4.4 Conclusions

On applying novel approaches to the cryopreservation of algae data generated has increased knowledge on the susceptibility of algae to injuries induced by cryoprotectant toxicity and the effects of dehydration. The toxicity of the vitrification solutions employed in this study has not enabled robust vitrification protocols to be developed for the algae. However, by employing encapsulation techniques it has been possible to freeze *E. gracilis*, an organism for which the development of robust preservation procedures has proven problematic, with a high degree of success.

Furthermore, during the course of this investigation there has been increasing evidence of the possibility of stresses causing both lethal and non-lethal injury and some indications that these may promote free radical mediated injury. The possibility of alginate acting in an "antioxidative" role, in addition, to limiting fracture events and structurally supporting the alga deserves further investigation. The importance of oxidative injury in cryopreserved *E. gracilis* is discussed in Chapter 7 and antioxidant responses in Chapter 8.

Chapter 7.

An investigation of free radical mediated cryoinjury in microalgae.

Contents. **Page No.**

7.1 Introduction

Cryopreservation involves many different steps, comprising, preparative culture manipulations, cryoprotection and low temperature treatments. It is imperative, for the development of improved protocols, to determine specific areas of the preservation procedure which cause damage, enabling preventative measures to be implemented to limit injury. The development of much of the current storage methodology for freshwater algae has, however, followed an empirical approach (Chapter 3). To further improve cryo-conservation protocols a major aim of this research has been the development of investigative tools for the study of cryoinjury in freeze-recalcitrant microalgae. Alternative methods employed to evaluate and investigate cryoinjury are microscopy, cryomicroscopy, flow cytometry and the oxygen electrode (Chapters 4, 5). These approaches have proven useful and have identified markers of both lethal and non-lethal injury (Fig. 4.2) as well as events culminating in cell mortality (Tables 4.1 - 4.5, Chapter 3). However, although these investigative techniques have indicated that oxidative injury/stress may be occurring they cannot be used to elucidate the biochemical basis of cryoinjury.

Considerable evidence exists to suggest that free radical damage occurs in tissues exposed to low and ultra-low temperature storage and that radical injury is exacerbated when suboptimal storage protocols are applied. Consequently, oxidative stress occurs in mammalian transplant organs exposed to low temperature storage (Fuller & Green, 1986; Cotterill *et al.*, 1989a) and free radical-mediated loss of organ function and tissue damage can be ameliorated by applying antioxidants and free radical scavenging agents (Whiteley *et al.*, 1992a,b; Pickford *et al.*, 1989; McAnulty & Huang, 1996; 1997; Green *et al.*, 1986b). In addition, some reports of "unexplained" changes in cryopreserved material may be due to suboptimal protocols which promote free radical injury (1.10). Free radical damage has also been implicated in the low temperature storage recalcitrance of germplasm from a wide range of higher plant species (Benson, 1990). Consequently, sensitivity to seed storage treatments has been associated with free radical processes (Hendry *et al.*, 1992; Magill *et al.*, 1994) and a diverse range of antioxidant treatments have been used to enhance the longevity of seeds stored at subzero temperatures (Benson, 1990; Basu & Dasgupta, 1978; Gorecki & Harman,

1987). Similarly, cryopreserved cells and tissues of higher plant species can undergo free radical mediated oxidative stress as evidenced by singlet oxygen formation, lipid peroxidation and free radical generation (Magill *et al.*, 1994; Benson & Withers, 1987; Benson & Noronha-Dutra, 1988; Benson *et al.*, 1994). Importantly, environmentally induced acclimation responses of higher plants to cold stress have been associated with the enhancement of antioxidant enzyme activities (Anderson *et al.*, 1995) knowledge of these natural protective mechanisms may be used to advantage by adapting them as cryoprotective strategies (Luo & Reed, 1997).

7.1.1 Free radicals and their importance in cryoinjury

Free radicals are a natural and intrinsic part of metabolic reactions in all living cells and one of their primary sources is oxygen (1.11.2). Although ubiquitous in photosynthetic organisms, oxygen at elevated levels may be deleterious promoting generation of toxic free radicals derived from oxygen (1.11.2). One of the primary reasons for the toxicity of free radicals is their highly reactive nature which promotes the production of cascades of damaging chain reactions in living tissue (Benson & Withers, 1987; Benson *et al.*, 1992a; Benson *et al.*, 1994; Benson & Noronha-Dutra, 1988). Reactive singlet oxygen can target membranes, nucleic acids and proteins promoting the production of radical species (1.11.2). In many biological reactions there is a close relationship between free radicals and transition metal ions, which are involved in both radical generation and scavenging, *e.g.*, the dismutation of superoxide (1.11.2). In the presence of a metal catalyst (copper or iron) the Haber-Weiss and Fenton reactions play an important role producing the (highly toxic) hydroxyl radical $^{\cdot}OH$ from hydrogen peroxide which may attack lipids, DNA, sugars etc. causing a wide variety of damage (Esterbauer *et al.*, 1988; Esterbauer *et al.*, 1990; 1.11.2).

Free radicals, and in particular those derived from oxygen, have been associated with the responses of plants to many external stresses including; dehydration, temperature extremes, heavy metals, herbicides, ionising radiation disease and senescence (Benson, 1990; Field, 1981; Field, 1984). Although these free radical responses are associated with stresses, which have been encountered throughout their evolution in response/adaptation to their environment, *e.g.*, drought, flooding, frost, *etc.*, plants also

encounter stresses during cryopreservation, *e.g.*, cryodehydration, the use of high molarity cryoprotectant solutions and temperature extremes (-196°C) (Fig. 7.1).

7.1.1.1 Exogenous antioxidants for the control of free radicals

To regulate free radical reactions and protect cells from oxidative damage cells commonly possesses antioxidant enzymatic and non-enzymatic defence systems (1.11.5). In addition, to the cellular antioxidants, it is possible to influence free radical levels by removing catalysts involved in their synthesis, or by the addition of exogenous antioxidants (1.11.9). DMSO and diludine have been reported to reduce H_2O_2 and MDA accumulation in the leaves of low temperature stressed tomato cultivars and thus increase frost hardiness (Kolosha *et al.*, 1988). In human platelets, a small improvement in the recovery of platelets after freezing, was achieved by pre-culturing cells in seleno-DL-methionine, an antioxidant which has been reported to inhibit lipid peroxidation in membranes, suggesting that oxidative damage could be implicated in freezing injury (Richter & Armitage, 1985). The iron chelating agent desferrioxamine (Keberle, 1967; Halliwell, 1989), may be employed to reduce hydroxyl radical-mediated injury. This drug is a potent iron cation chelating agent and it is routinely used to prevent the clinical and pathological generation of $^{\cdot}OH$ radicals in human medicine (Keberle, 1967; Halliwell, 1989). The possible role of iron-mediated free radical damage has recently been examined in cryopreserved blood cells (McAnulty & Huang, 1997) and desferrioxamine has been found to have beneficial effects in reducing free radical-mediated low temperature injury in mammalian transplant organs and tissues (Green *et al.*, 1986b; De Loecker *et al.*, 1997; Healing *et al.*, 1989; Gower *et al.*, 1989c). Post-thaw recovery of cryopreserved rice cells was enhanced in the absence of metal cations and presence of desferrioxamine (Benson *et al.*, 1995), thus, the drug may have a potential applications for the cryopreservation of algal protists.

7.1.2 Free radical assays

7.1.2.1 Invasive techniques

Measurements of lipid peroxidation induced by injury and stress may be evaluated by measuring the production of thiobarbituric acid-reactive substances (TBARS). Malondialdehyde (MDA) a breakdown product of lipid peroxides may be detected using thiobarbituric acid (TBA) assay and this technique was employed to detect the production of thiobarbituric acid-reactive substances in liver slices (Fraga *et al.*, 1988). The TBA assay may, also be employed to the investigation of lipid peroxidation in chill and freeze stressed material (Fig. 7.1) (Whiteley *et al.*, 1988a,b). In rabbit kidneys stored for 24 h. at 0°C significant increases in TBARS were detected (Cotterill *et al.*, 1989a). Furthermore, in tissue specimens stored at subzero temperatures, studies have indicated that sufficient lipid peroxidation may occur in rat liver homogenates in phosphate-buffered saline stored at -20°C to influence levels of malondialdehyde detectable using TBARS assays (Whiteley *et al.*, 1992a,b). Deterioration of animal tissue during storage due to lipid peroxidation has also been demonstrated, to a lesser extent, at -70°C, however, lipid peroxidation has not been detected at -196°C (Whiteley *et al.*, 1992a,b). This implies that specimens for free radical assays should be stored at -196°C to avoid the possibility of changes occurring during storage due to free radical activity. In addition, where living material is to be stored for prolonged periods, storage in LN will limit injury due to oxidative stress promoted by chilling and freezing the material. At temperatures used for cryopreservation (-196°C) normal cellular biochemical reactions will cease, as kinetic energy levels are too low to permit necessary molecular motion (Grout *et al.*, 1990). Under these conditions some chemical damage may occur due to free radical formation and ionising radiation, possibly damaging nucleic acids and in time affecting genetic stability (Grout *et al.*, 1990). However, at -196°C radicals formed during the freezing process are unlikely to be able to propagate the destructive "radical chain reactions" commonly associated with oxidative injury due to insufficient kinetic energy (Whiteley *et al.*, 1992a,b; Grout *et al.*, 1990). Instead, injury is likely to occur during warming and subsequent periods of recovery.

To accurately evaluate freeze-damage it is necessary to accompany the biochemical studies with viability and re-growth assessments. However, the TBARS assay, in common with many other biochemical investigative techniques, is in itself destructive (Harding & Benson, 1995). Further techniques for measuring lipid peroxidation and free radical activity include fluorescent determination of Schiff's bases and electron paramagnetic resonance (EPR) spectroscopy. The aldehyde breakdown products of lipid peroxidation (including MDA) are highly toxic as they can cross link with protein and DNA to form Schiff's bases (Lunec & Dormandy, 1979). Schiff's bases may be employed as an index of cold-induced oxidative stress in ischaemic organs (Fuller & Green, 1986; Green *et al.*, 1986b). Furthermore, fluorescent compounds with the spectral characteristics of "age related" Schiff's bases have been detected in plant tissue culture systems, permitting fluorescent determination of Schiff's bases to be employed for the study of lipid peroxidation in freezing injured plants (Harding & Benson, 1995; Benson & Roubelakis-Angelakis, 1992; 1994; Benson *et al.*, 1992b). In addition, to the detection of TBARS and products of their reaction with cellular structures, EPR spectroscopy may be employed for the detection of stable free radicals (Atherton *et al.*, 1993), however, results may prove difficult to interpret with high degrees of overlap between oxygen and carbon centred radicals (Goodman *et al.*, 1995).

However, for these destructive techniques it is necessary to perform simultaneous viability assessments to permit accurate interpretation of the biochemical data. The application of non-destructive techniques will remove this area of subjecture permitting viability assessments and biochemical assays to be performed on the same tissue.

7.1.2.2 Non-invasive techniques

The use of non-destructive methods for the assessment of freezing injury and recovery may be particularly useful because damage can be evaluated in the same tissues over an extended time-course and may be directly correlated with recovery events (Harding & Benson, 1995). This is the approach which will be explored in this project.

7.1.2.2.1 Non-invasive volatile hydrocarbon monitoring of cryoinjury and recovery

By recovering algal cells in gas-tight sampling vessels, "marker" volatiles may accumulate (Fig. 7.1). These volatiles may subsequently be withdrawn using a gas-tight syringe and measured using gas chromatography. In addition, to hydrocarbon volatiles produced due to lipid peroxidation (ethane, propane, pentane) and ethylene production due to alterations in membrane structure (Field, 1981; Field 1984; Corbineau *et al.*, 1990), DMSO may be introduced as a hydroxyl radical scavenger. DMSO employed in hydroxyl scavenging assays promotes evolution of the volatile hydrocarbon methane (Fig. 7.1) (Hebbel *et al.*, 1982).

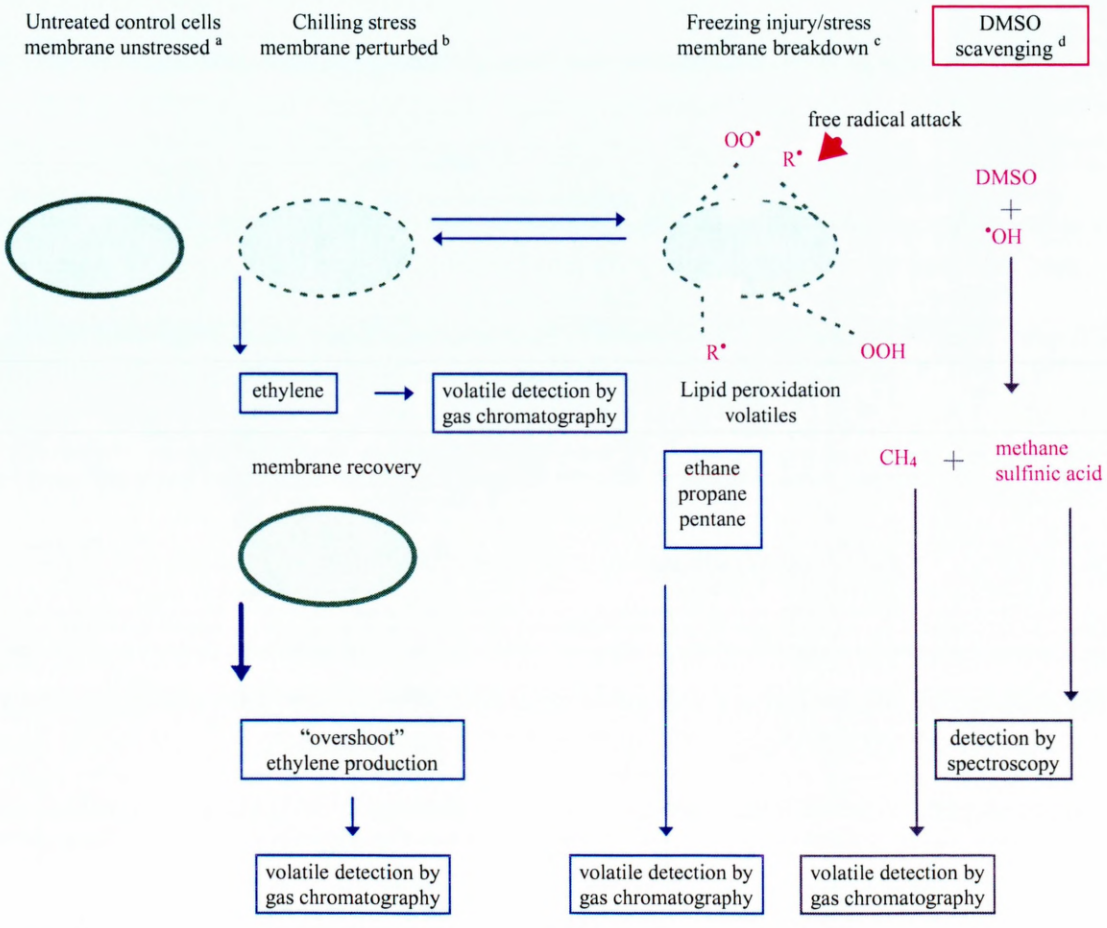


Figure 7.1 Detection of temperature-induced volatile hydrocarbons using gas chromatography.
Adapted from Harding & Benson (1995).

In Figure 7.1 the principles of volatile hydrocarbon production in cells exposed to chilling and freezing stresses are illustrated graphically. The figure illustrates the

production of volatiles under different treatments including: untreated control cells, chilling and freezing stresses cells and cells recovered in the presence of DMSO.

The untreated control cells do not produce any detectable changes in volatile hydrocarbons (Fig. 7.1[a]) (Field, 1981). Cells exposed to chilling injury may experience alterations in membrane conformation which may affect metabolic pathways involved in ethylene production, during subsequent recovery this may give rise to an "overshoot" of ethylene production (Fig 7.1[b]) (Field, 1981; Field, 1984; Corbineau *et al.*, 1990).

Production of the volatiles ethane, propane and pentane may be used as indicators of freezing stress and are associated with membrane breakdown products caused by free radical attack and lipid peroxidation (Fig. 7.1[c]) (Harber & Fuchigami, 1986a,b; Dillard & Tappel, 1979; Frank *et al.*, 1978; Fuller & Green, 1986). Furthermore, DMSO a powerful scavenger of the toxic hydroxyl free radical ($^{\cdot}OH$) may be introduced into the system to trap $^{\cdot}OH$ radicals (Fig. 7.1[c,d]). This process gives rise to several reaction products including $^{\cdot}CH_3$ which readily abstracts H to form methane (CH_4) which may be detected in head space samples using gas chromatography (Fig. 7.1[d]) (Hebbel *et al.*, 1982).

7.1.3 Objectives

The overall aim of this study is to explore the possibility that free radical damage may be a component of cryoinjury in microalgae. The role of oxidative stress in protist freeze-recalcitrance has not been previously investigated and as antioxidant treatments have been found to enhance cryo-tolerance in many other types of cellular material and organisms it is important to assess their potential for improving cryopreservation methodology in microalgae (Kolosha *et al.*, 1988; Richter & Armitage, 1985; McAnulty & Huang, 1996; Green *et al.*, 1986b; De Loecker *et al.*, 1997).

The research will investigate the application of the TBARS assay to the investigation of oxidative stress in *Euglena gracilis*, *Haematococcus pluvialis* and *Vaucheria sessilis*. Further studies will attempt to determine if lipid peroxidation is a potential source of injury in the alga *V. sessilis*.

E. gracilis has also been investigated in the context of the following research objectives:
1) Evaluating the potential of using dimethyl sulphoxide (DMSO) as a probe for the hydroxyl radical.
2) Using DMSO to detect the hydroxyl radical in *E. gracilis* and assess its potential involvement in cryoinjury.
3) Developing a profile of free radical production throughout a cryopreservation protocol by employing the non-destructive monitoring of methane in algal head space samples by gas chromatography.
4) Determining which of the components of the cryopreservation protocol are the most damaging and appraising strategies which may be appropriate for enhancing cryo-tolerance in the alga.

A further objective of the study will be to explore the possibility of using the iron chelating agent desferrioxamine, to reduce hydroxyl radical-mediated cryoinjury in cryopreserved *E. gracilis* (Keberle, 1967; Halliwell, 1989).

7.2 Materials and methods

7.2.1 Organisms and culture regimes

Cultures selected for study are detailed in 2.1 (*Haematococcus pluvialis* Flotow CCAP 34/8, *Euglena gracilis* Klebs CCAP 1224/5Z and *Vaucheria sessilis* (Vaucher) De Candolle ex Collins CCAP 745/1C). Filaments of the xanthophytic alga *Vaucheria sessilis* were prepared as described in 4.2.1. Organism culture regimes and recovery conditions were as described in 2.2-2.3.

7.2.2 Cryopreservation procedures

Cryoprotectant solutions were always added to cell suspensions, to avoid excessive toxic shock. Cryoprotectant chemicals employed in these studies were dimethylsulphoxide (DMSO) or methanol (Sigma, USA). Final concentrations of 5 or

10% (v/v) of cryoprotectant were used throughout, with a 5 or 15min. exposure period at room temperature (RT/20°C) or at 0°C prior to cooling to subzero temperatures.

Vials containing 0.5ml of algae/cryoprotectant were frozen employing controlled two-step cooling using a Planer Kryo 10 programmable freezer (Planer, UK) followed by a direct plunge into LN (2.4). Cooling rates of -1°C $min.^{-1}$ and -0.5°C $min.^{-1}$ were employed. Vials were cooled to -35°C or -60°C and held at their intermediate temperature for either 15 and 30 min. periods, prior to being plunged directly into LN.

Vials were thawed using a two-step protocol by which vials were first allowed to slowly warm while being held in the air for 1 min. followed by more rapid warming in a pre-heated 40°C water bath by direct immersion in a pre-heated water bath at 40°C. All vials were agitated until the last ice crystals had melted (Day *et al.*, 1997). The thawed material was then aseptically transferred to appropriate fresh, sterile, medium (2.2), and used in the viability assays as outlined in 2.5. All experiments were repeated in triplicate and errors are expressed as standard errors of mean.

7.2.3 Flow cytometry

Flow cytometry was carried out on treated cells after a predetermined recovery period of either 24 h. or 48 h. using a FACStar Plus flow cytometer (Becton Dickinson, UK) as described in 2.5.8, using the vital stain fluorescein diacetate (FDA) prepared in methanol (2.5.2.1).

7.2.4 Volatile hydrocarbon analysis by gas chromatography

7.2.4.1 Vial preparation

Identical glass vials with an approximate volume of either 4 ml or 15 ml (Suplico, USA) were used throughout. The volume of the glass vials were accurately determined as 4.8ml ± 0.00ml and 16.37ml ± 0.02ml respectively. Vials were then sealed with air tight silicon-teflon septa (Suplico) and sterilised with their silicon-teflon lids in place by autoclaving (10 Bar). Immediately post sterilisation the vials were opened and allowed

to vent for 2 h. in a laminar flow bench. This ensured no volatiles were retained in vials which were due to autoclaving.

7.2.4.2 Cell preparation

Euglena gracilis cell suspensions were cultured under standard conditions (2.2-2.3). Cells from a single culture were divided 24 h. prior to cooling and pre-cultured, under standard conditions, in identical volumes of EG:JM media or EG:JM media supplemented with 10 $mg.l^{-1}$ desferrioxamine (DesF). Immediately prior to chilling non-centrifugation (untreated control) cells (2ml) were removed to sterile recovery tubes. The remaining *E. gracilis* cultures were dispensed (2ml) into sterile centrifuge tubes and concentrated by centrifugation and resuspended in 0.25ml EG:JM. Cells were then treated using a standard cryopreservation protocol (7.7.2). At all preparation steps care was taken to ensure identical 2ml samples of cell suspension were dispensed. In addition, care was also taken to ensure that during each manipulation cell suspensions were neither diluted nor concentrated. Furthermore, care was taken to assure that each individual 2ml cell suspension was exposed to an identical number of transfers. The cell suspensions were rapidly dispensed in a single procedure using an Eppendorf multipette 4780 (Eppendorf, Germany). Post-thaw, cryoprotectants, if present, were removed by centrifugation and all cells were re-suspended in 2ml of the appropriate recovery media. Cells were recovered in either EG:JM media, EG:JM supplemented with 1% (v/v) DMSO and EG:JM supplemented with 1% (v/v) DMSO and 10 $mg.l^{-1}$ desferrioxamine.

For logistical purposes sufficient cell samples were prepared to permit the removal of triplicate samples at predetermined stages in the cryopreservation protocol for later analysis: Samples corresponding to the following treatment steps were collected: untreated control cells which were not centrifuged at any stage during preparation, cells which were centrifuged to concentrate them prior to cryopreservation, concentrated cells which had been exposed to cryoprotectant [10% (v/v) methanol], concentrated, cryoprotected cells which had been cooled at -0.5°C $min.^{-1}$ to -10°C, concentrated, cryoprotected cells which had been cooled at -0.5°C $min.^{-1}$ to -60°C and concentrated, cryoprotected cells which had been cooled at -0.5°C $min.^{-1}$ to -60°C, held for 30 min. prior to being plunged into LN.

7.2.4.3 Preparation of samples for gas chromatography

Pre-prepared sterile vials (7.2.4.1) were vented in a laminar flow bench for 2 h. immediately prior to the addition of tissue. Cell suspensions (2ml) were placed in pre-prepared sterile glass vials (4ml or 15ml) and sealed with air tight silicon-teflon septa. The volume of the glass vials had previously been determined, allowing accurate calculation of head space above the cell suspension. The time was recorded at the point of sealing the vials, this permitted the calculation of the duration between sealing the vial and injecting the head space samples into the gas chromatography and permitted the determination of rate of volatile production.h.$^{-1}$. The vial head space was assayed by gas chromatography for volatile hydrocarbons at recovery intervals of either 4 h., 24 h. or 48 h. during 24 h., 7 day or 14 day recovery periods respectively, with venting of the vials following sampling.

7.2.4.4 Gas chromatography

Head space samples (1ml) were injected into a Perkin-Elmer 8310 Gas Chromatograph (Perkin-Elmer, USA), fitted with a 2m Poropak Q column and a hydrogen/air flame ionisation detector. The volatile hydrocarbons were separated using a temperature program with an initial oven temperature of 35°C and a predetermined isothermal time. Two gas chromatographic programs were prepared, programme 1) was employed for the detection of methane, ethane, ethylene and propane and had an isothermal time of 6 min., programme 2) was employed for the detection of methane and had an isothermal time of 2 min. The injector and detector temperatures were 200°C and 225°C respectively. A carrier gas (nitrogen gas) flow rate of 10ml min^{-1}, was employed throughout. The gas chromatograph was pre-calibrated and the volatile hydrocarbon peaks (methane, ethane, ethylene and propane) identified using standard ppm gas mixtures. Identified peaks and volatile concentrations (ppm) were stored in the in built computerised data handling facility. At all stages, appropriate controls were incorporated into the analyses to determine the presence of any background volatiles evolved from DMSO, media, vessels, septa, cryoprotectants and the laboratory atmosphere.

Empty control vials (9 vials) were also included in the experimental design and the volatile hydrocarbon levels were recorded immediately after the vials were sealed (3 vials) and at the start (3 vials) and end (3 vials) of the experimental gas chromatographic analysis. These data sets were employed as background volatile levels. Where necessary these background levels of volatiles were subtracted from the cell suspension sample data (Benson & Withers, 1987).

Head space samples (1ml) were withdrawn using a gas-tight syringe, after the head space had been thoroughly mixed by flushing the syringe out several times (5 times) without withdrawing the needle. Two, 1ml injections were taken form each vial. After head space sampling, samples were returned to a laminar flow bench and aerated for 20 min. before resealing. Recovery was performed under standard culture conditions.

Volatile production.h.$^{-1}$ was calculated for data corrected for background volatile hydrocarbons (methane). Data was expressed as parts per million (ppm) volatile (as designated by the calibration gases) per 10^6 cells and represented as means with standard errors of mean, n = 6.

7.2.5 The fluorimetric thiobarbituric (TBARS) assay

Flourimetric determination of thiobarbituric reactive substances (TBARS) was performed on extracts of *Euglena gracilis*, *Haematococcus pluvialis* and *Vaucheria sessilis*. In all cases experimental procedures were carried out rapidly maintaining parity between samples following the procedure described by Fraga *et al.* (1988).

Stock solutions of 3% (w/v) Lauryl sulphate (sodium dodecyl sulphate) (SDS), Sigma L-4509 (Sigma, USA) and 0.1N (0.1 molar) Hydrochloric acid (HCl) were prepared in advance in distilled water. Fresh stock solutions of 10% (w/v) phosphotungstic acid, Sigma P-4006 (Sigma, USA) and 0.7% (w/v) thiobarbituric acid (TBA) were prepared immediately prior to the assay in distilled water.

Assays were run in conjunction with malondialdehyde (MDA) (malonaldehyde bis[dimethyl acetal] [Sigma, T-1642, (Sigma, USA)]) standards. MDA was prepared as a 10mM stock in 1% (v/v) sulphuric acid and allowed to hydrolyse for 2 h. prior to preparing standards: 2μM, 4μM, 6μM, 8μM, 10μM, 15μM and 20μM MDA in distilled water. MDA stock and standards were prepared fresh immediately prior to the assay.

Cells samples, or MDA standards (0.5ml), were then added to pre-labelled centrifuge tubes to which the reactants were added, as follows: 0.5ml of 3% (w/v) SDS, 2.0ml of 0.1N HCl, 0.3ml of 10% (w/v) phosphotungstic acid and 1ml of 0.7% (w/v) TBA. Centrifuge tubs were then transferred to a pre-heated, boiling (100°C) water bath located in a fume cupboard and boiled for exactly 30 min. The boiled tubes were then transferred directly to an ice bucket and held on ice for 10 min. (this procedure cools the reaction mixture and stops the reaction). Butan-1-ol (5ml) was then added to the cooled tubes to extract the TBA/MDA complex (a pink coloured complex). Tubes were then vortexed for 15 sec. and centrifuged for 10 min. in a refrigerated centrifuge at 5000 rpm. This procedure separated the butan-1-ol containing the pink TBA/MDA complex from the reaction constituents. The top organic layer was then removed and its fluorescence emission recorded. Fluorescence was recorded at 555nm using and excitation wavelength of 515nm against a solvent blank (Harding & Benson, 1995).

7.2.6 ANOVA

The flow cytometry results were analysed separately in a two way balanced ANOVA with treatments and recovery procedure as main effects (Zar, 1996). In addition, a one way ANOVA was performed for recovery procedures (Zar, 1996). Data were $\log_e$ transformed to increase normality of the residuals. All calculations were performed using Minitab v.11.2 (Minitab, USA).

7.3. Results

7.3.1 *Euglena gracilis*

7.3.1.1 Malondialdehyde, thiobarbituric acid-reactive substances

Identification of the TBA/MDA complex was hindered by interference from other complexes formed with cellular constituents and cryoprotective additives. The organic layer commonly developed an orange pigmentation obscuring the pink coloured TBA/MDA complex (at 555nm). Due to these additional pigments in the organic layer TBARS was not considered to permit accurate assaying for lipid peroxidation products in *E. gracilis*. In addition, considerable interference, due to coloured complexes, was identified when alginates and vitrification solutions were present. However, no interference was detected in EG:JM, JM media controls or cryoprotectant controls for DMSO [5% (v/v)] or methanol [10% (v/v)].

7.3.1.2 Gas chromatography

Gas chromatography proved to be an effective and accurate method for assaying volatile hydrocarbon markers. DMSO scavenging of hydroxyl groups was effectively employed for the investigation of free radical activity by monitoring methane production. This method was selected in preference to the TBARS assay.

Analysis of the head space when *E. gracilis* was recovered in 15ml vials permitted monitoring of hydrocarbon volatiles, however, the head space was found to be excessively large to permit accurate measurement of small changes in methane due to DMSO scavenging of hydroxyl radicals over 24 h. periods. These preliminary findings did, however, indicate that monitoring of changes in the concentration of methane in the head space could be achieved. With this established, all subsequent experiments were performed using 4ml vials, permitting accurate measurement of changes in methane concentration at less than 1 ppm.

During a 14 day recovery period, assaying for volatile hydrocarbons at 48 h. intervals, no ethane, ethylene or propane peaks were detected. However, elevated methane levels were detected in vials containing cells in EG:JM media supplemented with DMSO. In addition, changes in methane concentration were also monitored at 4 h. intervals during a 24 h. recovery period. These results were, difficult to interpret due to the low levels of methane produced during 4 h. interval and diurnal effects (due to the light:dark regime followed under a standard culture regime).

Detailed assaying for changes in the methane concentration were performed over a 5 day recovery, assaying for changes in methane concentrations at 24 h. intervals (Figs. 7.2-7.4). Trace levels of methane were detected in head space samples taken from media, empty vials and cryoprotectant controls [methanol 10% (v/v)] and were attributed to background methane. Cells recovered in EG:JM did not display a large increase in methane evolution with respect to treatment [Fig. 7.2 (light blue)]. However, cells recovered in media supplemented with 1% (v/v) DMSO produced a significant increase in methane where cultures had previously experienced stresses [Fig. 7.2 (dark blue)]. The correlation between increased methane evolution in the presence of DMSO post-stress indicates that DMSO was acting as a free radical scavenger during the duration of the recovery [Fig. 7.2 (dark blue)]. Where cells were recovered in EG:JM supplemented with 1% (v/v) DMSO and 10 $mg.l^{-1}$ desferrioxamine, significantly lower ($P < 0.004$) levels of methane were evolved 48h.-120h. after thawing [Figs. 7.2, 7.3 (yellow)].

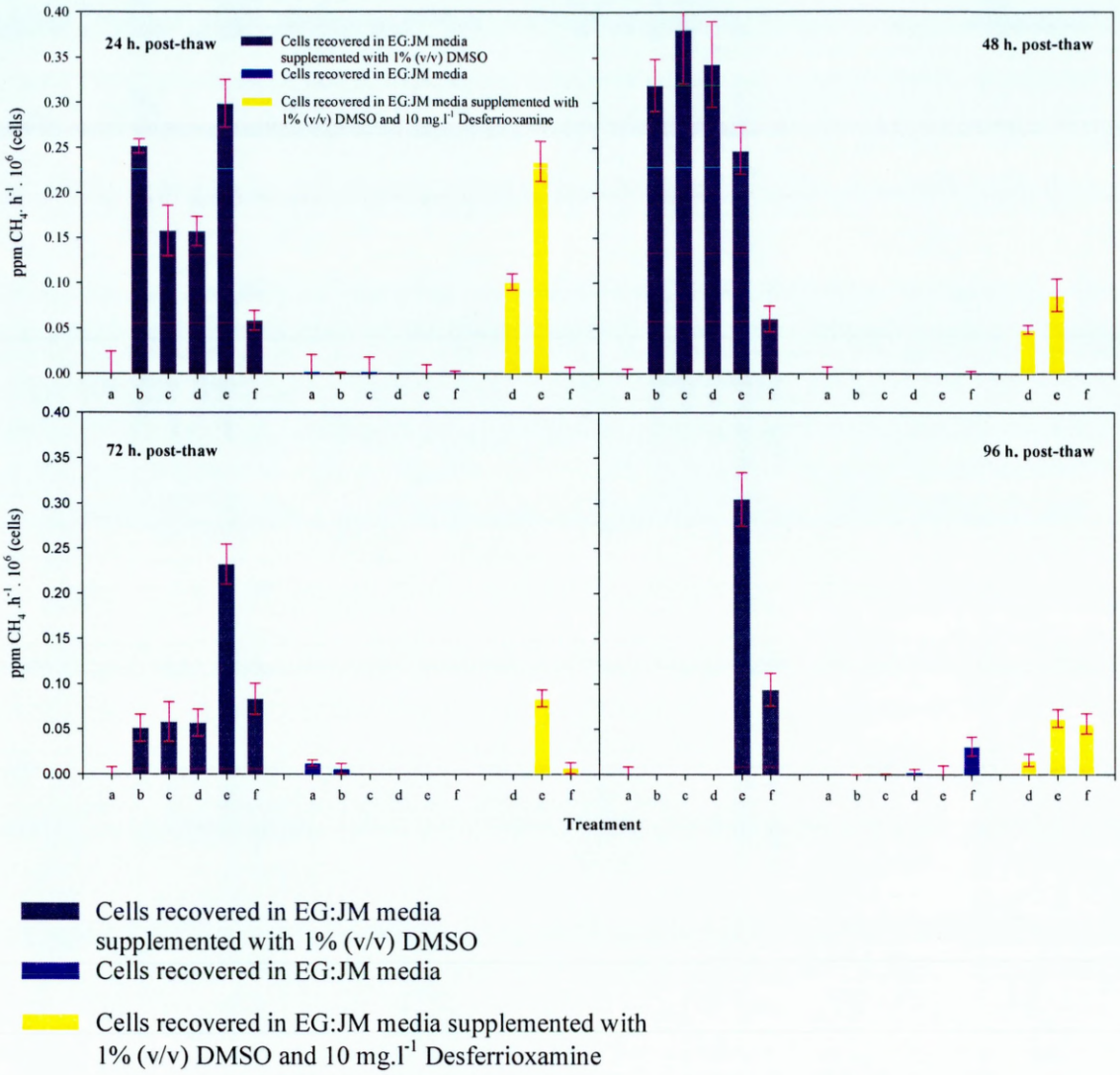


Figure 7.2 The effect of different recovery media on CH_4 production for *Euglena gracilis*, assessed by gas chromatographic analysis during a 5 day recovery period.

(a) Untreated control, (b) Cells centrifuged for 1 min., (c) Cells exposed to cryoprotectant at 0°C (10% (v/v) methanol) for 15 min., (d) Cells control cooled to -10°C (e) Cells control cooled to -60°C and held for 30min, (f) Cells plunged into LN from -60°C two-step thaw 1min in air followed by immersion in a 40°C water bath, All material was thawed using a simple two-step protocol.
n = 6, errors are expressed as standard errors of mean.

For cultures recovered in the presence of DMSO, [1% (v/v)] (dark blue) peak levels of methane evolution.h.$^{-1}$ for cultures exposed to non-lethal stresses were detected 48 h. after thawing (Fig. 7.2). During the preceding 24 h. recovery period the highest recorded levels of methane production.h.$^{-1}$ were for cultures which had been centrifuged (b) and those which had been control cooled to -60°C, after 48 h. of recovery the all levels of methane evolution.h.$^{-1}$ were elevated and the highest levels were for cells which had

been exposed to the cryoprotectant [methanol 10% (v/v)] (c) and cultures which had been control cooled to -10°C (d) (Fig. 7.2). During subsequent 24 h. recovery periods levels of methane evolution.h.$^{-1}$ decreased in cultures which had been exposed to non-lethal stresses [Cells centrifuged for 1 min. (b), Cells exposed to cryoprotectant at 0°C (10 % (v/v) methanol) for 15 min. (c) and Cells control cooled to -10°C (d)] (Figs. 7.2, 7.4, 7.5). After 96 h. of recovery under standard conditions, methane levels in these cultures had returned to untreated control levels and were largely attributed to background levels of methane [Fig. 7.2, (a-d), 7.5 (purple, dark blue, yellow, cyan)]. Levels of methane production remained high throughout the time course for the culture which had been control cooled to -60°C [Figs. 7.2, 7.3 (e), 7.5 (light blue)].

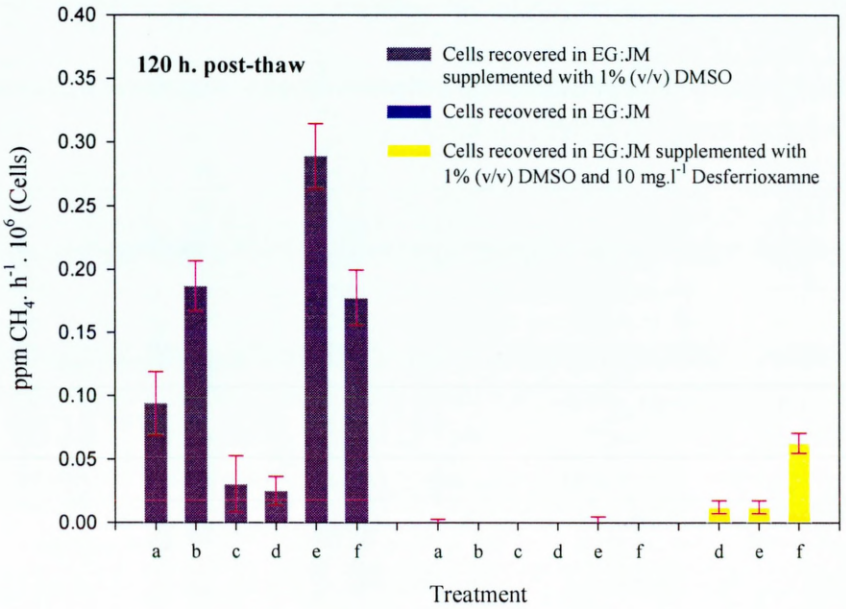


Figure 7.3 The effect of different recovery media on CH_4 production for *Euglena gracilis*, assessed by gas chromatographic analysis during a 5 day recovery period.

(a) Untreated control, (b) Cells centrifuged for 1 min., (c) Cells exposed to cryoprotectant at 0°C (10 % (v/v) methanol) for 15 min., (d) Cells control cooled to -10°C (e) Cells control cooled to -60°C and held for 30min, (f) Cells plunged into LN from -60°C two-step thaw 1min in air followed by immersion in a 40°C water bath, All material was thawed using a simple two-step protocol.
n = 6, errors are expressed as standard errors of mean.

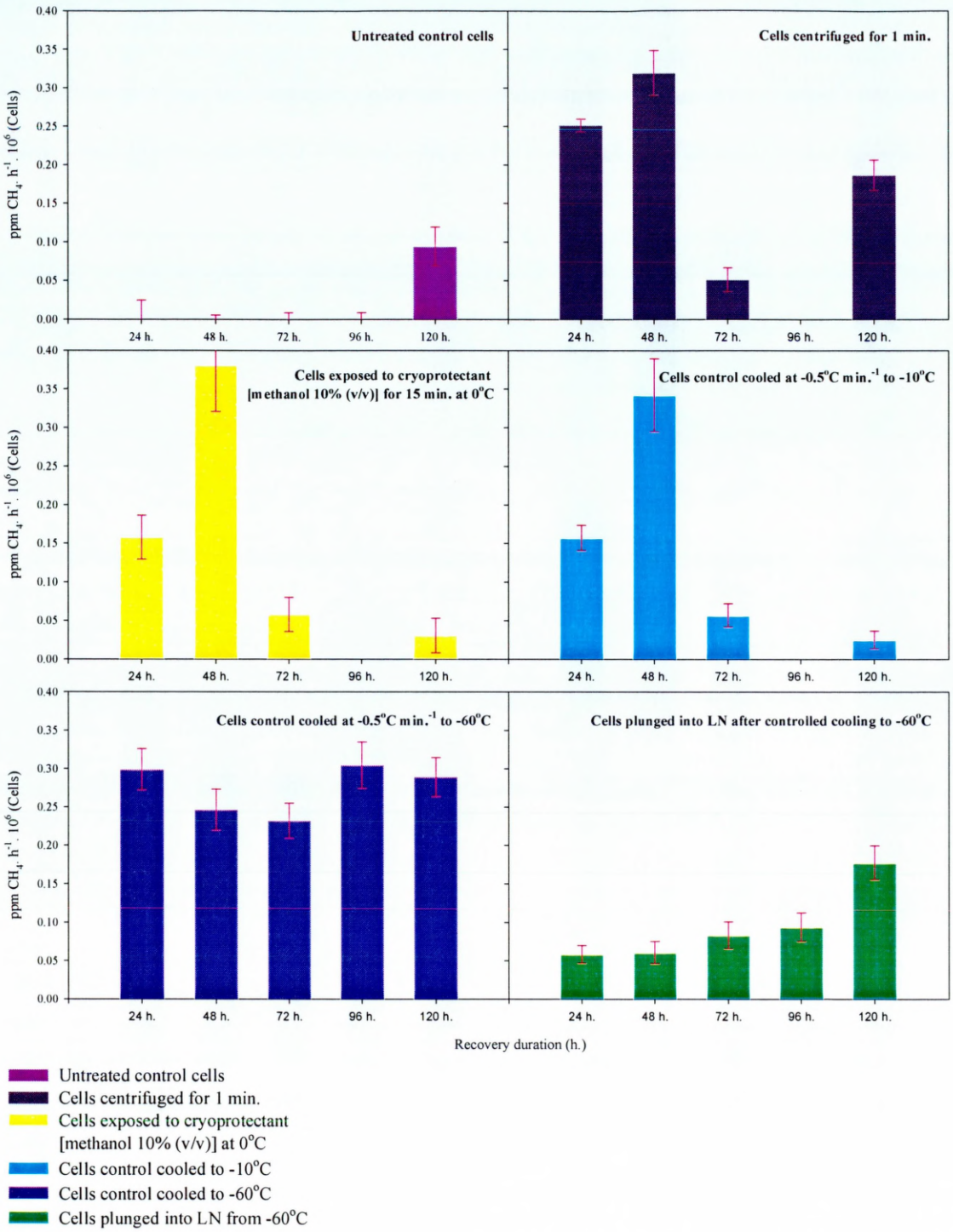


Figure 7.4 The effect of the duration of recovery on CH_4 production for *Euglena gracilis*., recovered in EG:JM media supplemented with 1% (v/v) DMSO, assessed by gas chromatographic analysis at 24 h. intervals during a 5 day recovery period.

(a) Untreated control, (b) Cells centrifuged for 1 min., (c) Cells exposed to cryoprotectant at 0°C (10 % (v/v) methanol) for 15 min., (d) Cells control cooled to -10°C (e) Cells control cooled to -60°C and held for 30min, (f) Cells plunged into LN from -60°C two-step thaw 1min in air followed by immersion in a 40°C water bath, All material was thawed using a simple two-step protocol.
n = 6, errors are expressed as standard errors of mean.

Cells recovered in EG:JM supplemented with 1% (v/v) DMSO and 10 $mg.l^{-1}$ desferrioxamine had significantly elevated levels of methane evolution during the first 24 h. of recovery for cultures which had been exposed to controlled cooling to -10°C or -60°C, however, levels of methane evolution were significantly reduced during subsequent 24 h. recovery periods [Fig. 7.2 (yellow d,e)]. Methane evolution was always significantly lower than levels detected for cultures recovered in EG:JM supplemented with 1% (v/v) DMSO without desferrioxamine present [Figs. 7.2, 7.3 (dark blue, yellow)]. Following 120 h. of recovery methane evolution.$h.^{-1}$ begins to rise in all treatments recovered in the presence of DMSO [1% (v/v)] including untreated control cultures [Fig. 7.3 (dark blue)].

Viability levels for *E. gracilis* in vials used for monitoring changes in methane concentration during continuous 24 h. periods during a 5 day recovery protocol were determined by vital staining with fluorescein diacetate (FDA) and counted using flow cytometry (Fig. 7.5). From these viability assessments it was determined that cells were not lethally injured by the centrifugation step, exposure to the cryoprotectant [methanol 10% (v/v)] or controlled cooling to -10°C at -0.5°C $min.^{-1}$. In addition, recovery in EG:JM, EG:JM supplemented with 1% (v/v) DMSO and EG:JM supplemented with 1% (v/v) DMSO and 10 $mg.l^{-1}$ desferrioxamine did not affect post-treatment viability levels, viability was > 99% ± 0.1% for all treatments and recovery procedures (Fig. 7.5). Viability after exposure to -60°C and LN were > 50% and > 40% respectively (Fig. 7.5).

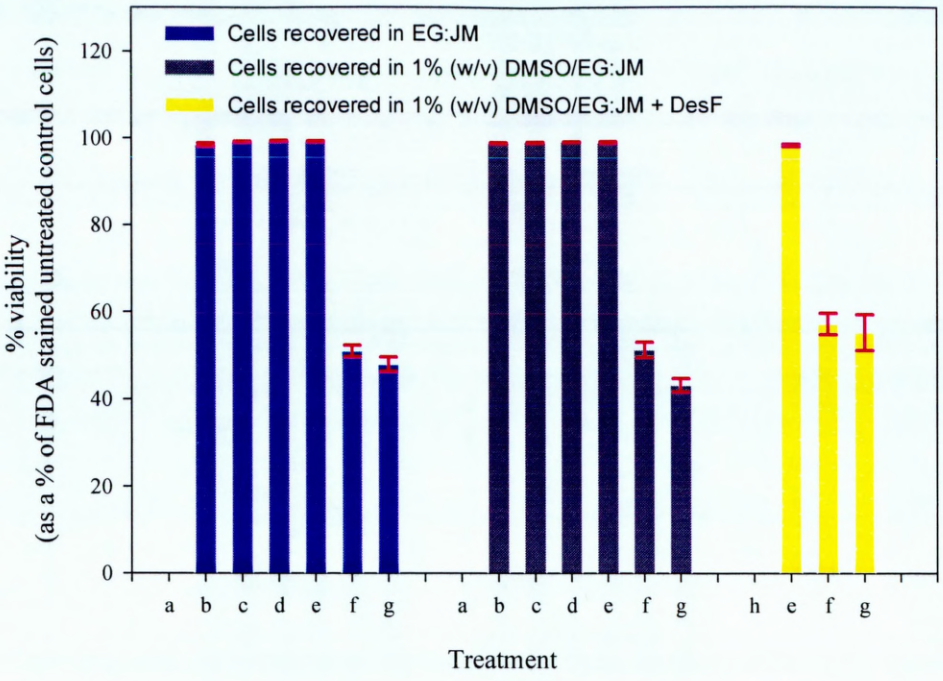


Figure 7.5 The effect of different recovery media on post-treatment viability in *Euglena gracilis*, assessed by FDA staining 8 days post-thaw.

(a) Unstained untreated control, (b) Untreated control, (c) Cells centrifuged for 1 min., (d) Cells exposed to cryoprotectant at 0°C (10 % (v/v) methanol) for 15 min., (e) Cells control cooled to -10°C (f) Cells control cooled to -60°C and held for 30min, (g) Cells plunged into LN from -60°C two-step thaw 1min in air followed by immersion in a 40°C water bath, (h) Unstained cells after controlled cooling to -10°C. All material was thawed using a simple two-step protocol.
n = 3, errors are expressed as standard errors of mean.

Significant differences were detected between recovery in EG:JM, EG:JM supplemented with 1% (v/v) DMSO and EG:JM supplemented with 1% (v/v) DMSO and 10 $mg.l^{-1}$ desferrioxamine when a one way ANOVA was performed on data which had been log_e transformed to increase normality. An increase in the level of post-thaw viability was detected when cells were recovered with desferrioxamine present ($F_{2,6}$ = 5.26, $P < 0.05$). A two way balanced ANOVA with treatment (exposure to -10°C, -60°C and LN) and recovery (EG:JM, EG:JM supplemented with 1% (v/v) DMSO and EG:JM supplemented with 1% (v/v) DMSO and 10 $mg.l^{-1}$ desferrioxamine) indicated that both the treatment and recovery procedure had a significant effect on post-exposure viability but were not synergistic. Treatments were significantly different ($F_{2,12} = 8.07$, $P < 0.01$) and the recovery procedure was significantly different ($F_{1,2} = 6.07$, $P < 0.05$) (Table

7.1). Table 7.1 represents the values for mean viability with ± errors on which the ANOVA was performed.

Table 7.1 Data tested using two way ANOVA representing the % viability of *Euglena gracilis* as a % of untreated FDA stained cells, data is reverse $\log_e$ transformed and shows geometric means and reverse transformed errors.

Treatment/recovery[a] conditions	Mean	Positive error	Negative error
Control cooled to -60°C[e] (EG:JM[b])	51	1.34	1.31
Plunged into LN from -60°C[f] (EG:JM[b])	48	1.65	1.59
Control cooled to -60°C[e] (DMSO[c])	51	1.79	1.73
Plunged into LN from -60°C[f] (DMSO[c])	43	1.54	1.49
Control cooled to -60°C[e] (DesF[d])	57	2.56	2.45
Plunged into LN from -60°C[f] (DesF[d])	55	4.46	4.13

[a] Treatment step and recovery procedure employed
[b] Cells recovered in EG:JM media
[c] Cells recovered in EG:JM media supplemented with 1% (v/v) DMSO
[d] Cells recovered in EG:JM media supplemented with 1% (v/v) DMSO and 10 $mg.l^{-1}$ desferrioxamine
[e] Cells control cooled at -0.5°C $min.^{-1}$ to -60°C and held for 30min,
[f] Cells plunged into LN from -60°C
All material was thawed using a simple two-step protocol.
n = 3, errors are expressed as standard errors of mean.

All viability assessments were performed after completion of the gas chromatographic study of volatile hydrocarbons, 8 days after thawing.

Viability levels, as determined by FDA staining, for cells exposed to -60°C and LN and recovered in EG:JM were 51.1% ± 1.3%, 48.0% ± 1.6% respectively. Cells exposed to

-60°C and LN and recovered in EG:JM supplemented with 1% (v/v) DMSO and 10 mg.l^{-1} desferrioxamine and 57.3% ± 2.4% and 55.4% ± 4% respectively (Fig. 7.5).

7.3.2 *Haematococcus pluvialis*

7.3.2.1 Malondialdehyde, thiobarbituric acid-reactive substances

Identification of TBA/MDA complex was hindered by interference from other complexes formed with cellular constituents. Interference was particularly noticeable when assays were performed using aplanospore stage cultures. The organic layer commonly developed an orange pigmentation obscuring the pink coloured TBA/MDA complex. Due to the additional interference from cellular pigments in the organic layer TBARS was not considered to permit accurate assaying for lipid peroxidation products in *H. pluvialis*.

7.3.3 *Vaucheria sessilis*

7.3.3.1 Malondialdehyde, thiobarbituric acid-reactive substances

Identification of TBA/MDA complex was possible for *V. sessilis* and a pink organic layer was readily formed without interference from other complexes. No interference due to the JM media or the cryoprotectant methanol was found. However, the difficulty in culturing large quantities of *V. sessilis* created its own problems and it was found that although TBA/MDA complexes could be derived from *V. sessilis* tissue the quantities obtained were insufficient to permit detailed analysis of lipid peroxidation due to different degrees of chilling and freezing stress (thus assay sensitivity was problematic).

TBARS assays performed on filaments of *V. sessilis* which had been subjected to different mechanical stresses were performed (Fig. 7.6). Elevated TBA/MDA complexes were detected in all cases where filaments had been exposed to mechanical injury [Fig. 7.6 (b-d)].

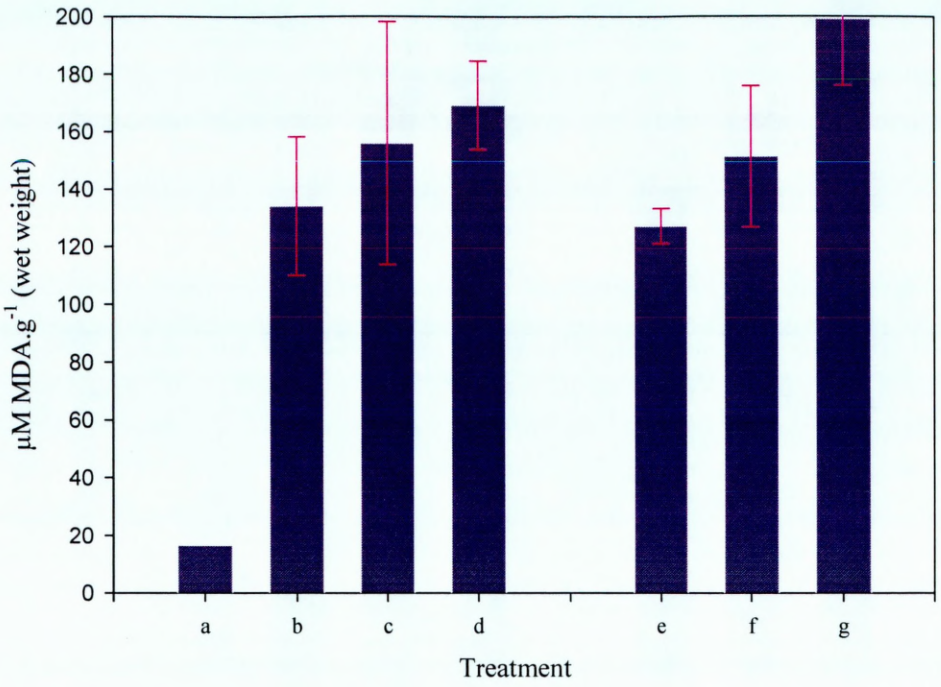


Figure 7.6 Fluorimetric determination of thiobarbituric reactive substances (TBARS) in *Vaucheria sessilis* filaments after exposure to different stresses.

(a) Untreated control, (b) Filaments sectioned by teasing apart, (c) Filaments sectioned by cutting into 12mm lengths, (d) Filaments exposed to cryoprotectant at 20°C (5 % (v/v) DMSO) for 15 min., (e) Filaments control cooled to -35°C and held for 30min., (f) Cells plunged into LN from -35°C, (g) Filaments plunged directly into LN from 20°C, without cryoprotectant.
n = 3, errors are expressed as standard errors of mean.

7.4 Discussion

Investigations to explore the possibility that free radical damage may be a component of cryoinjury in microalgae has permitted the development of reliable investigative techniques and has identified markers of injury and tissue trauma, which may be induced by exposure to freezing temperatures. Dimethyl sulphoxide (DMSO) has been demonstrated to be a valuable probe for identification of the hydroxyl radical (Hebbel *et al.*, 1982). In addition, desferrioxamine has been illustrated to reduce hydroxyl production in cryopreserved *E. gracilis*, and may be employed to reduce hydroxyl radical-mediated cryoinjury. This has previously been reported in similar studies performed on low-temperature stored mammalian tissues (Gower *et al.*, 1989c) and cryopreserved higher plant systems (Benson *et al.*, 1995).

7.4.1 Free radical attack in freeze/chill injured cells

In studies on non-protistan systems rewarming of frozen tissue has previously been associated with the simultaneous generation of free oxygen radicals (Bhaumik *et al.*, 1995). Free radical production and lipid peroxidation in tissues maintained at low temperatures and following low temperature storage has been reported for mammalian systems (Green *et al.*, 1986a; Whiteley *et al.*, 1988a,b; 1992a,b; Fuller, 1988; Fuller *et al.*, 1988). In addition, lipid peroxidation has been measured using the thiobarbituric acid (TBA) assay for malondialdehyde (MDA) in frozen/thawed higher plant tissues (Benson *et al.*, 1992a).

In the low temperature storage of tissues, injury has been linked to the breakdown of lipid membranes and the liberation of free fatty acids (Bhaumik *et al.*, 1995). On rewarming, free fatty acids are metabolised and this may be a source of oxygen free radicals (Bhaumik *et al.*, 1995). Furthermore, iron has been identified as playing a major role in the formation of oxygen radicals, the production of lipid peroxides and specific free radical degradation of proteins (Benson *et al.*, 1995). This is commonly involved in the production of active oxygen species via the Fenton reaction, most commonly through the reactions of iron with H_2O_2 (1.11.9) producing via a one electron reduction the hydroxyl anion and the hydroxyl radical ($^{\cdot}OH$) (Benson *et al.*, 1995). The superoxide radical $O_2^{\cdot-}$ is also closely associated with the Haber-Weiss reaction, leading to reduction of Fe^{3+} salt and the further production of hydroxyl radicals ($^{\cdot}OH$) from H_2O_2 and Fe^{2+} which may then participate in Fenton reactions (Pierre, 1995; Benson, 1990; Benson *et al.*, 1995). It can be seen that the damaging role of transition metals in lipid peroxidation is due to their ability to catalyse the formation of the highly toxic hydroxyl radical, the peroxides resulting from hydrogen abstraction by the lipid peroxide radical can also participate in iron mediated reactions which may ultimately lead to the production of toxic aldehydes, malondialdehyde and hydroxyalkenals (Benson *et al.*, 1995). This study has investigated both radical production and lipid peroxidation in the algae *E. gracilis*, *H. pluvialis* and *V. sessilis*.

7.4.2 Thiobarbituric acid-reactive substances

The TBA assay was selected for these investigations because it permits the direct measurement of levels of thiobarbituric reactive substances (including MDA) within tissue extracts (Fraga *et al.*, 1988). MDA is a readily detectable product of lipid peroxidation and forms a coloured complex with TBA which can be detected by either spectroscopy or fluorimetry (Harding & Benson, 1995). However, TBA has also previously been reported to form complexes with sugars, polyols and aldehydes (Harding & Benson, 1995). Many of these compounds may be incorporated within tissue culture media and/or employed in a cryoprotective capacity. As such it is important to routinely determine if interference with the detection of MDA is occurring. Although these compounds if present may form coloured complexes, they have been reported to fluoresce at a different wavelength (out with the 600-532nm absorbance range used to detect MDA) from those of MDA complexes (Harding & Benson, 1995). Further reports have indicated that TBA coloured complexes may be formed with additional peroxidation breakdown products (which absorb in the range of 450-550nm) indicating that the TBA assay is not entirely specific to MDA (Kosugi & Kikugawa, 1989). It is possible that the interference complexes encountered in TBARS assays employing *E. gracilis* and *H. pluvialis* may not have fluoresced in the same wave length as the TBA/MDA complexes. However, the dominance of these interference complexes over the TBA/MDA complex discouraged further use of this technique for these organisms and particularly the investigation of lipid peroxidation in vitrified or encapsulated material, where, significant coloured complexes were detected in cryoprotectant controls.

Lipid peroxidation in *V. sessilis* was readily investigated by employing the fluorimetric TBARS assay (Fig. 7.6). However, difficulties in culturing sufficient biomass of *V. sessilis*, to permit detailed investigations of lipid peroxidation during a cryopreservation protocol, whilst performing a concomitant assessment of viability, limited the application of this assay technique in *V. sessilis*. Results did however indicate that significant levels of MDA could be detected in filaments which had been exposed to chilling/freezing injury and mechanical membrane injury (either as a result of cutting or bruising during sectioning, or as a result of ice crushing) (Fig. 7.6). Increases in

MDA/TBA complexes were in excess of 600% for filaments exposed to chilling/freezing and/or mechanical injury.

7.4.3 Gas chromatography

Gas chromatography was employed to detect volatile hydrocarbons in gas-tight sampling vessels and the recovery of tissues in these vessels permitted the accumulation of volatile "marker" compounds of lipid peroxidation (Harber & Fuchigami, 1986a,b). This non-destructive technique allowed the evaluation of damage in the same tissues over an extended recovery period which could then be correlated with "real time" recovery events (Harding & Benson, 1995). The technique was employed to construct a profile of the production of "marker" volatile hydrocarbons during different stages of preparative manipulations, cryoprotection, chilling, freezing/thawing and recovery for *E. gracilis*. "Marker" volatiles selected for investigation were: ethylene a "marker" of membrane perturbation due to chilling injury (Field. 1981; 1984; Corbineau *et al.*, 1990), ethane, propane and pentane "markers" produced due to the breakdown of membranes following free radical attack and lipid peroxidation (Dillard & Tappel, 1979; Frank *et al.*, 1978) and methane a "marker" volatile for $^{\cdot}OH$ produced when DMSO is introduced to the system to scavenge $^{\cdot}OH$ (Hebbel *et al.*, 1982).

7.4.3.1 Non-destructive volatile hydrocarbon monitoring by gas chromatography

Analysis of head space samples when *E. gracilis* was recovered in 15ml vials permitted monitoring of hydrocarbon volatiles. However, the volume of the head space in the 15ml vials was determined to be excessively large to permit accurate measurement of small changes in volatiles during 24 h. periods. The preliminary findings did, however, indicate that monitoring of changes in the concentration of methane in the head space was possible. To improve accuracy and sensitivity, all subsequent experiments were performed using 4ml vials, which permitted accurate measurement of changes in methane concentration at less than 1 ppm. During a 14 day recovery period, assaying for volatile hydrocarbons at 48 h. intervals, no ethane, ethylene or propane peaks were detected. However, elevated methane levels were detected in vials containing cells in EG:JM media supplemented with DMSO. In addition, changes in methane

concentration were also monitored at 4 h. intervals during a 24 h. recovery period. These results were difficult to interpret due to the low levels of methane produced during the 4 h. intervals and diurnal effects introduced by the standard light:dark culture regime (2.3).

7.4.3.2 Profile of volatile hydrocarbon production

7.4.3.2.1 Ethylene production

Production of ethylene, a hormonal marker of stress in higher plants, may be detected in cells exposed to chilling injury. Cells may experience alterations in membrane conformation which may affect the metabolic pathways involved in ethylene production, which may give rise to an "over shoot" of ethylene production during subsequent recovery (Fig 7.1[b]) (Field, 1981; Field, 1984; Corbineau *et al.*, 1990). It has previously been reported that ethylene production may be inhibited by free radical scavengers and copper chelators (Apelbaum *et al.*, 1981a,b). The standard culture conditions for *E. gracilis* employed EG:JM media, this medium contains EDTA, a copper and iron chelator (Table 2.7). In addition, many cultures were recovered in media supplemented with 1% (v/v) DMSO a free radical scavenger. Potent inhibition of ethylene production has also been linked to uncouplers of oxidative phosphorylation (Apelbaum *et al.*, 1981a,b).

Damage to chloroplasts by low temperatures and freezing have been reported and they have been described as exhibiting a number of changes (Wise *et al.*, 1983; Ginsburger-Vogel *et al.*, 1992; Morris *et al.*, 1985; Heber *et al.*, 1971). Heber *et al.* (1971) and Santarius (1987) have reported that thylakoid membranes damaged by freezing may experience inactivation of photophosphorylation (1.11-1.11.1). In Chapter 5 inhibition of the photosynthetic capacity, attributed to thylakoid/chloroplasts stress/injury, was described for *E. gracilis* cells which had been exposed to low and subzero temperatures. Furthermore, in cells which had been exposed to -60°C and whose photosynthetic capacity was measured during the duration of the subsequent recovery, photosynthetic capacity was seen to increase from 17% ± 3% 24 h. (post-thaw) to 48% ± 8% 48 h. (post-thaw) (Fig. 5.5). This was attributed to non-lethal injury which inhibited the cells

photosynthetic capability during the first 24 h. of recovery. In addition, studies comparing photosynthetic capacity and viability (assessed by FDA staining and flow cytometry), indicated that there may a degree of continued photosynthetic inhibition (Chapter 5). These results and studies present the possibility of prolonged inhibition of oxidative phosphorylation. Together, the inhibitory effects of EDTA, DMSO and uncoupling of oxidative phosphorylation may account for the apparent lack of ethylene evolution in *E. gracilis*. In addition, ethylene production, which requires membrane integrity, may be greatly reduced, or absent, in cells which have incurred damage to their ethylene evolving systems. This response has previously been reported for cells frozen without cryoprotectant (*i.e.*, cells mortally injured by intracellular ice formation) (Benson & Withers, 1987). Ethane production may be employed as an indicator of the progress of freezing-induced mortality with ethylene production ceasing as ethane production (a marker of cell breakdown) increases (Benson & Withers, 1987). However, some studies have reported ethane to be a minor volatile product of lipid peroxidation (Benson & Withers, 1987).

7.4.3.2.2 Profile of lipid peroxidation volatile hydrocarbons

Ethane, propane and pentane are volatile hydrocarbons which have previously been employed as "markers" of membrane breakdown due to free radical attack and lipid peroxidation (Dillard & Tappel, 1979; Frank *et al.*, 1978). Lipid peroxidation, a complex process whereby unsaturated lipid undergoes reaction with molecular oxygen in living tissues, has been reported to be mediated by malfunctioning metabolic processes (Benson & Withers, 1987; Freeman, 1984). Impairment of metabolic processes may lead to oxyradical production particularly at electron transport sites in mitochondria and chloroplasts (Benson & Withers, 1987; Wise *et al.*, 1983), in addition, inactivation of phosphorylation has been identified in frozen thylakoid membranes (Heber *et al.*, 1971; Santarius, 1987). However, cryoprotective compounds may protect thylakoid membranes against inactivation of phosphorylation (Heber *et al.*, 1971).

In studies performed on higher plants marked production of pentane and propane with a slight increase in ethane production was identified in callus cultures pre-grown in the presence of manitol (Benson & Withers, 1987). This may be due to peroxidative injury

due to changes in the callus vacuole. Vacuole size is metabolically and nutritionally controlled, with decreased vacuole size having a corresponding increased lipid content (Finkle *et al.*, 1985). Preculture in the presence of mannitol or sorbitol was reported to cause a proportion of cells in a sycamore suspension cultures to shrink and display structural alterations including an decrease in cell wall thickness and an appearance of being more densely cytoplasmic (Pritchard *et al.*, 1986). *E. gracilis* was not exposed to an osmotically challenging pre-culture regime and viability studies have indicated that cell are not lethally injured by exposure to the cryoprotectant methanol (Chapter 5). However, although hydrogen peroxide [an initiator of lipid peroxidation (Halliwell, 1982)] was detected in cultures exposed to the cryoprotectant none of the selected volatile hydrocarbons due to lipid peroxidation were detected. This may be due to the chemical composition of *E. gracilis* promoting production of volatiles other than those selected for study (Benson & Withers, 1987).

Evolution of pentane and isobutane was observed to significantly increase in callus cultures exposed to a conventional cryopreservation regime (Benson & Withers, 1987). Neither of these volatiles were assayed for in the present study, however, unidentified peaks were observed and it would be interesting to progress the study to investigate these, as yet, unidentified peaks. Lipid peroxidation may result in the production of numerous end products dependant upon the nature of the lipid substrate, the presence of metal cations, free radical scavenging and oxygen tension (Benson & Withers, 1987; Frank *et al.*, 1978). It is possible that a different range of lipid peroxidation markers will be produced by *E. gracilis*. In cryopreserved plant tissue cultures lethally injured cells were found to have increased propane evolution, however, ethane was reported to be completely absent (Benson & Withers, 1987).

In the cryomicroscopic investigations described in Chapter 4, only a small proportion of *E. gracilis* cells (5%) frozen at the optimum cooling rate of -0.5°C $min.^{-1}$ were observed to undergo lethal intracellular ice nucleation (Table 4.1). In the investigations detailed above, *E. gracilis* cells were cooled at the optimum rate and it could be expected that only a small proportion of the cells would experience lethal freezing injury due to intracellular ice nucleation. Furthermore, in the studies performed by Benson & Withers (1987) lethal injury was induced in cells by freezing cells in the absence of

cryoprotectant. However, in all studies performed on *E. gracilis* cryoprotectants were present and may be assumed to have provided a degree of protection to all cells (Heber *et al.*, 1971). Benson & Withers (1987) also reported that the distribution of these volatiles was unaffected in cells exposed to non-lethal injury. It is therefore possible that under the optimum cryopreservation protocol for the cryopreservation of *E. gracilis* employed for these studies, only a small proportion of the cells would have been subjected to the gross injury employed by Benson & Withers (1987) to induce propane production. However, it is worth noting that considerable evidence exists for high levels of $^{\cdot}OH$ within cells exposed to both lethal and non-lethal stress (7.4.3-7.4.3.2) and $^{\cdot}OH$ has previously been reported to be a initiator of lipid peroxidation (Halliwell, 1982).

Decompartmentalisation, cell death and gross physical injury have been suggested to be mediated by breakdown mechanisms different from those encountered in living tissues (Benson & Withers, 1987). Loss of compartmentalisation attributed to freezing injury, may result in degradation of phospholipids by lytic enzymes (Stout *et al.*, 1980; Rajashekar *et al.*, 1979, Yoshida, 1979a,b). Studies have also demonstrated that disruption of membrane compartmentalisation, structure and function at lethal freezing temperatures may contribute to freeze recalcitrance (Singh & Miller, 1985). Many of these lethal injuries have been attributed to dehydration rather than gross disruption by intracellular ice nucleation (Singh & Miller, 1985; Weist & Steponkus, Williams *et al.*, 1981; Williams and Hope, 1981) (1.11.1). Loss of compartmentalisation would therefore be expected to promote the production of a different range of breakdown products. The absence of markers of lipid peroxidation (ethane, propane) may indicate that injury in *E. gracilis* was due to decompartmentalisation.

The absence of detectable lipid peroxidation markers in *E. gracilis* may therefore be attributed to a series of complex effects including: presence of cryoprotectants, different lipid compositions, limited lethal injury due to intracellular ice nucleation in cells frozen at the optimum cooling rate. In *V. sessilis*, however, mechanical injury was able to promote the accumulation of lipid peroxidation products, freezing injury also promoted the accumulation of high levels of lipid peroxidation products and may be attributed to injury to cellular organelles including the mitochondria and chloroplasts which were discussed in Chapter 4.

7.4.3 Use of DMSO as a probe for monitoring hydroxyl radicals

DMSO was introduced into recovery media to act as a probe for hydroxyl radicals. DMSO a powerful scavenger of the toxic hydroxyl free radical ($^{\cdot}OH$) traps $^{\cdot}OH$ radicals (Fig. 7.1[c,d]), giving rise to several reaction products including $^{\cdot}CH_3$ which readily abstracts H to form methane (CH_4) which may be detected in head space samples using gas chromatography (Fig. 7.1[d]) (Hebbel *et al.*, 1982).

Preliminary gas chromatographic investigations established that low levels of methane could be detected in head space samples taken from media controls, empty vials and cryoprotectant controls [methanol 10% (v/v)]. These low levels of methane were identical in each set of vials and were attributed to background concentrations of methane. It was also determined that recovery in EG:JM supplemented with 1% (v/v) DMSO did not affect cellular viability levels, viability was > 99% ± 0.1% of the untreated control for DMSO exposure control and non-lethal treatments [Fig. 7.5 (light blue b, c, d, e)]. Furthermore, the pre-preparation of vials (venting of vials following autoclaving and prior to use) for head space analysis appears to have removed any sources of contamination due to the vials themselves, and assaying of empty vials immediately after sealing and 24 h. later did not detect any changes in volatile levels.

7.4.3.1 Profile of hydroxyl radical production

A significant increase in methane evolution was detected in stressed cultures recovered in EG:JM media supplemented with 1% (v/v) DMSO permitting gas chromatography to be employed as an effective and accurate method for assaying volatile hydrocarbon markers of hydroxyl radical production. DMSO was employed to scavenge hydroxyl groups permitting the investigation of free radical activity by monitoring methane production. Detailed assaying for changes in the methane concentration were performed during a 5 day recovery, assaying for changes in methane concentrations at 24 h. intervals (Figs. 7.2-7.4).

Cells recovered in EG:JM did not display a large increase in methane evolution with respect to treatment [Fig. 7.2 (light blue)]. This was attributed to the absence of the hydroxyl scavenger DMSO. However, cells recovered in media supplemented with 1% (v/v) DMSO produced a significant increase ($P < 0.004$) in methane where cultures had previously experienced stresses [Fig. 7.2 (dark blue)]. The correlation between increased methane evolution in the presence of DMSO post-stress indicates that DMSO was acting as a free radical scavenger during the duration of the recovery [Fig. 7.2 (dark blue)] see also Benson & Withers (1987). Further confirmation that methane was due to oxidative stress was the ability of the iron chelator desferrioxamine to reduce/inhibit methane production (1.11.9).

The production of hydroxyl radicals via the Haber-Weiss reaction is kinetically too slow to account for the biological generation of $^{\cdot}OH$ radicals without the presence of catalytic metal salts (*e.g.*, iron and copper) (Nivière & Fontecave, 1995). The Haber-Weiss reaction can be catalysed by iron in the ferric form, reducible by superoxide anions. Alternatively ferrous iron may catalyse the production of hydroxyl radical by transferring electrons to hydrogen peroxide, the Fenton reaction (Nivière & Fontecave, 1995). Desferrioxamine is a powerful Fe^{3+} chelator is therefore able to prevent the production of the hydroxyl radicals, by limiting availability of Fe^{3+} (1.11.9). Where cells were recovered in EG:JM supplemented with 1% (v/v) DMSO and 10 $mg.l^{-1}$ desferrioxamine, a significantly lower level ($P < 0.004$) of methane was evolved 24 h. - 120 h. after thawing [Figs. 7.2, 7.3 (yellow)].

However, elevated levels of methane evolution were detected during the first 24 h. of recovery for cultures which had been exposed to controlled cooling to -10°C or -60°C and were recovered with desferrioxamine present [Fig. 7.2 (24 h. post-thaw, yellow d,e)]. This was likely to be due to the experimental design, in which all cultures were exposed to cryoprotocol treatment steps in the absence of DMSO and desferrioxamine and were only subsequently recovered in media supplemented with these chemicals. Thus, no hydroxyl radical scavengers (DMSO) or iron chelator was present during treatments to limit or influence free radical production. During the subsequent 24 h. recovery period hydroxyl radicals already within the culture would have been scavenged by the DMSO, leading to methane evolution. However, following subsequent incubation

in the presence of desferrioxamine, methane evolution reduced and returned to close to background levels (Fig. 7.2). The introduction of desferrioxamine into recovery media was, therefore, able to limit hydroxyl radical production and may therefore be expected to reduce injury due to toxic hydroxyl radicals [Figs. 7.2, 7.3 (yellow)].

A profile of *E. gracilis* response to non-lethal stresses over a 96 h. duration was clearly evident. Viability levels for *E. gracilis* in vials used for monitoring changes in methane concentration during continuous 24 h. periods during a 5 day recovery protocol were determined by vital staining with fluorescein diacetate (FDA) and counted using flow cytometry (Fig. 7.5). From these viability assessments it was determined that cells were not lethally injured by the centrifugation step, exposure to the cryoprotectant [methanol 10% (v/v)] or controlled cooling to -10°C at -0.5°C $min.^{-1}$. In these non-lethally stressed cultures there was an immediate post-stress increase in the evolution of methane which peaked after 48 h. before rapidly declining to close to control levels (72 h. post-thaw) [Fig. 7.4 (dark green, dark blue, yellow, cyan)]. Methane evolution for cells which were subjected to the single stress of centrifugation (dark blue) increased more rapidly during the first 24 h. than in cultures which had been exposed to the additional stress of cryoprotectant exposure and/or cooling to -10°C. This delay in methane evolution in cells exposed to cryoprotectant at 0°C (10 % (v/v) methanol) and for cells control cooled to -10°C in cryoprotectant is most likely due to *E. gracilis* being exposed to low temperatures which influenced the rate of radical production (Fig. 7.4). During the subsequent 24 h. recovery period levels of methane evolution.$h.^{-1}$ decreased in cultures which had been exposed to non-lethal stresses [Cells centrifuged for 1 min. (b), Cells exposed to cryoprotectant at 0°C (10 % (v/v) methanol) for 15 min. (c) and Cells control cooled to -10°C (d)] (Figs. 7.2, 7.4, 7.5). After 96 h. incubation under standard conditions, methane levels in these cultures had returned to untreated control levels and were largely attributed to background levels of methane [Fig. 7.2, (a-d), 7.5 (purple, dark blue, yellow, cyan)]. From these results it appears that cultures may experience non-lethal stress due to preparative culture manipulations prior to cryopreservation. These stresses, in isolation, do not affect cellular viability and cultures were determined to have fully recovered during a 96 h. incubation period. However, these non-lethal stresses may affect viability levels in frozen cultures by acting in a cumulative or synergistic fashion (Fig. 7.4, 7.5).

The profile of methane production in cultures which had been control cooled to -60°C [Figs. 7.2, 7.3 (e), 7.5 (light blue)] indicated that methane evolution remained high throughout the time course (120 h.). This indicates a considerable degree of stress in these cultures which may promote substantial hydroxyl radical production during recovery. Flow cytometry investigations have indicated that > 40% of cells may experience lethal injury during a freeze/thaw cycle to -60°C (Fig. 7.5, Table 7.1). Significantly lower levels of methane evolution were detected in cultures which had been exposed to LN [Fig. 7.4 (light green)]. The difference in methane evolution between cultures exposed to LN and -60°C may be a result of gross lethal injury in cells exposed to LN, limiting hydroxyl radical production, with only the healthy viable cells being intact and capable of producing hydroxyl radicals. In cultures exposed to -60°C a proportion of intact, but ultimately non-viable cells may be present which are a significant source of hydroxyl radicals. By reducing or eliminating the stresses/injury which promote hydroxyl radical production in these cells, it may be possible to significantly increase post-thaw viability levels in cryopreserved *E. gracilis*.

Following 120 h. of recovery methane evolution.h.$^{-1}$ began to rise in all treatments recovered in the presence of DMSO [1% (v/v)] including untreated control cultures [Figs. 7.3 (dark blue), 7.4 (120 h.)]. This increased methane evolution was attributed to hydroxyl production as a consequence of natural senescence. In senescence, free radical involvement has been suggested and they may be derived from oxygen senescence processes and other forms of oxidative damage (Benson, 1990; Magill *et al.*, 1994; Merzlyak & Hendry, 1994) (1.11.2).

These studies have indicated that elevated levels of H_2O_2 may be present in stressed cells. This has been inferred by the detection of increased levels of CH_4 (due to the scavenging of $^{\cdot}OH$ by DMSO) and it has been hypothesised that the primary route for the production of $^{\cdot}OH$ is via the iron-catalysed production of $^{\cdot}OH$ from H_2O_2 (following the effective inhibition of $^{\cdot}OH$ production by the iron chelator desferrioxamine) (Halliwell, 1982; 1974; 1989). A primary route for the production of H_2O_2 in cells is via the activity of superoxide dismutase (Halliwell, 1982). Furthermore, in studies on

chilling stress in *Arabidopsis thaliana*, "oxidative stress" was identified as an important aspect of chilling injury, which may, in its initial stages be exacerbated by increased levels of superoxide dismutase activity (Burdon *et al.*, 1994). A mode of cryoinjury in *E. gracilis* may therefore involve increased superoxide dismutase activity, which could thereby increase the availability of H_2O_2, which could contribute to the generation of $^{\cdot}OH$ and the initiation of subsequent lipid peroxidation (Burdon *et al.*, 1994).

7.4.3.2 Use of an exogenous iron chelator to control hydroxyl radical production

The close relationship between free radicals and transition metal ions in many biological reactions (Haber-Weiss and Fenton reactions) include both radical generation and scavenging, *e.g.*, the dismutation of superoxide (1.11.2). A product of these reactions, the highly toxic hydroxyl radical $^{\cdot}OH$, may attack lipids, DNA, sugars *etc.* causing a wide variety of damage (1.11.2). In addition, Fe^{2+} may promote the accumulation of astaxanthin in algae (1.5.5). Furthermore, *E. gracilis* has been reported to show an absolute requirement for iron for growth (Ishikawa *et al.*, 1993a).

Addition of exogenous iron chelating compounds, *e.g.*, desferrioxamine has previously been employed to suppress oxidative stress in mammalian transport organs subjected to cold storage (Benson *et al.*, 1995; Fuller & Green 1986). Desferrioxamine, a powerful Fe^{3+} chelator is able to prevent harmful transition metal chemistry, suppressing oxidative stress encountered in rice (Benson *et al.*, 1995) and it has been successfully used to reduce lipid peroxidation in cold-treated rabbit kidneys (Green *et al.*, 1986b; Gower *et al.*, 1987). Recovery in EG:JM supplemented with 1% (v/v) DMSO and 10 $mg.l^{-1}$ desferrioxamine did not affect post-treatment viability levels, viability was > 99% ± 0.1% of the untreated control for treatments without a lethally injurious treatment step [Fig. 7.5 (yellow e)]. Viability after exposure to -60°C and LN were > 50% and > 40% respectively in all cases (Fig. 7.5).In these studies the significantly higher viability levels obtained where cultures exposed to LN were recovered in EG:JM supplemented with 1% (v/v) DMSO and 10 $mg.l^{-1}$ desferrioxamine indicated that the application of exogenous antioxidants and radical mediators may offer considerable scope for enhancing post-thaw viability levels in cryopreserved algae and may assist in cryopreserving presently freeze-recalcitrant algae ($F_{2,6} = 5.26$, $P < 0.05$). Although the

increase in post-thaw viability was relatively low it is worth noting that viability was assessed 8 days following thawing, during which time cultures would have undergone a number of cell divisions. However, *E. gracilis* has an absolute requirement for iron for growth, with iron depletion causing arrest of cell division (Ishikawa *et al.*, 1993a). If cellular division was arrested in cultures recovered in the presence of desferrioxamine then the difference in post-thaw viability between cultures with and without desferrioxamine present may be greater than data suggests. In this study and in the study by Benson *et al.* (1995) desferrioxamine was employed to suppress oxidative stress at critical points of cryoinjury and during the initial stages of recovery. Desferrioxamine would then be removed immediately following these periods to minimise iron limitation effects on regrowth.

7.4.3 Conclusions

The TBARS assay was only employed with limited success for the investigation of oxidative stress in *Euglena gracilis* and *Haematococcus pluvialis*. The limiting factor in the application of TBARS to the investigation of cryoinjury is interference by coloured complexes other than TBA/MDA, originating from algal pigments, notably in the aplanospore stage of *H. pluvialis* cells. A further limitation of TBARS, and all destructive assays, is the requirement to run concurrent viability studies and/or multiple cultures to permit changes during the duration of recovery to be investigated. TBARS was successfully employed to the investigation of lipid peroxidation in *Vaucheria sessilis*, however, the slow generation/growth rate of this alga proved problematic for the design and implementation of more detailed investigations due to problems with assay sensitivity.

Dimethyl sulphoxide (DMSO) was effectively employed as a probe for hydroxyl radicals and the non-invasive study of free radical involvement in cryoinjury in *E. gracilis*. The recovery of the alga at a low concentration of DMSO [1% (v/v)] was effective in permitting the monitoring of methane evolution due to the scavenging of hydroxyl radicals, without affecting viability, or reducing the effectiveness of the cryoprotectant protocol though the use of a suboptimal cryoprotectant. This is an important development in the use of DMSO as a probe for hydroxyl radicals in

organisms which are intolerant to exposure to high concentrations of DMSO, or where DMSO has not proven to be an effective cryoprotectant.

It was possible to develop a profile of free radical production throughout a cryopreservation protocol by employing the non-destructive monitoring of methane by gas chromatography in the head space of sealed vials containing algae. Although, each stage in the cryopreservation protocol introduced a degree of stress, only exposure to freezing temperatures induced a prolonged stress response > 48 h. (Fig. 7.4). This response could be associated with treatments which had resulted in cell mortality which have been reported in previous Chapters. Free radical damage is almost certainly an important component of cryoinjury in microalgae, and confirms the hypotheses that radical mediated events were affecting cell viability and recovery as discussed in previous chapters. In addition, considerable evidence exists for promoting the use of the iron chelating agent desferrioxamine, to reduce hydroxyl radical-mediated cryoinjury in cryopreserved *E. gracilis* (Keberle, 1967; Halliwell, 1989; Benson *et al.*, 1995; Gower *et al.*, 1989c; Green *et al.*, 1986b; Healing *et al.*, 1989). It would be interesting to perform further studies focusing on the investigation of additional, as yet, unidentified volatiles which were observed during the course of this study and the use of further exogenous antioxidants. Chapter 8 will investigate antioxidant responses to chilling and freezing within the algae themselves and may indicate which exogenous antioxidants could be most effectively employed to enhance cryo-tolerance.

Chapter 8.

An investigation of the role of antioxidants in cryotolerance and cryoinjury.

Contents. **Page No.**

8.1 Introduction

In tissues, free radical species are constantly produced as a result of oxidative metabolism and electron transport. Free radical species include oxygen radicals and oxygen containing intermediates of oxyradical reactions and are highly reactive, *e.g.*, activated singlet oxygen and hydrogen peroxide (Benson *et al.*, 1992a,b). Essential cellular free radical reactions are tightly controlled, however, during stress or as a result of disease and ageing, free radicals are cytotoxic and may attack vital cellular structures including: membranes, organelles and macromolecules. These oxidative stresses are discussed in detail in Chapter 1 (1.11.2-1.11.4) and Chapter 7.

In plant callus cultures the accumulation of species associated with free radical injury including lipid peroxidases, malondialdehyde and lipoxygenase activity have been identified (Cutler *et al.*, 1989). In studies on callus cultures an inverse relationship was found between increases in lipid peroxidation products and antioxidant status (decrease in catalase and peroxidase activities) (Cutler *et al.*, 1989). These findings prompted Cutler *et al.* (1989) to conclude that the highly tissue culture recalcitrance of cereal mesophyll protoplasts may be attributed to oxidative stress. Furthermore, the tissue culture of woody plant species has proven problematic, with high levels of phenolics promoting deleterious oxidation events during *in vitro* manipulations (Benson & Roubelakis-Angelakis, 1992). Preliminary studies of oxidative stress in micropropagated Sultanina demonstrated fluctuations in the antioxidant enzymes superoxide dismutase (SOD) and catalase, and also, decreases in markers of oxidative stress were accompanied by increased SOD activity (Benson & Roubelakis-Angelakis, 1992).

Free radical mediated damage has also been implicated in the physiological deterioration of mammalian transplant organs following low temperature storage (Janjic *et al.*, 1996; Schon *et al.*, 1993; Fuller *et al.*, 1988; Green *et al.*, 1986a,b). These mammalian examples of free radical mediated damage and reports of the detection of lipid peroxidation products in cryopreserved plant cultures (Benson *et al.*, 1992a; Benson & Noronha-Dutra, 1988; Benson *et al.*, 1995) have resulted in free radical mediated damage being proposed as a factor contributing to the freeze-recalcitrance of

many plant and mammalian tissues 1.11.4. Endogenous antioxidant status may therefore be an important variable in mediating oxidative injury in cryopreserved tissues. The possibility of oxidative stress contributing to the comparative freeze-recalcitrance of *Euglena gracilis* was proposed in Chapter 7. The link between oxidative stress and fluctuations in antioxidant status may indicate that the antioxidant potential of a cell could influence its ability to successfully recover from a freeze-thaw cycle.

Stresses, including low temperature stress, have been linked to the formation of free radicals in many systems (Benson, 1990; Fuller, 1988; Fuller *et al.*, 1988). During the course of this study algae have demonstrated a series of stress responses to cryoprotectant exposure, chilling and freezing (Chapters 3-5). Furthermore, in Chapter 7 direct evidence of free radical production was presented for *E. gracilis*. Although the essential free radical reactions are tightly controlled by antioxidants. The antioxidants and fluctuations in their activity/concentrations may also prevent oxidative injury induced during stress or damage (1.11.6).

This may be achieved through free radical scavenging by both exogenous and endogenous antioxidants (1.11.9). The use of an exogenous compound (10 $mg.l^{-1}$ desferrioxamine) to inhibit oxidative stress was discussed in Chapter 7. Additionally, in studies on low temperature preservation, addition of scavengers of oxygen derived free radicals (SOD) and catalase for the regulation of H_2O_2 have been demonstrated to yield improved function in rabbit kidneys (Bennett *et al.*, 1987). It is, therefore, important to investigate fluctuations in endogenous free radical scavenging systems and their role in cryotolerance and cryoinjury.

8.1.1 Endogenous antioxidants

In order to protect aerobic cells from oxidative injury, all organisms have developed an extensive array of antioxidants which protect them from damage by free radicals. Antioxidant defences may be achieved via both enzymatic and non-enzymatic reactions (1.11.5). The majority of these antioxidant systems are inter-dependent and function in recycling protective compounds as well as directly regulating toxic species (Scott, 1997;

Benson, 1990; Halliwell, 1982). These interactions and recycling reactions are discussed in detail below.

The non-enzymatic antioxidant vitamin E (α-tocopherol) directly scavenges free radicals and has been identified as being important in protecting chloroplast membranes in plants (Cay & King, 1980). Vitamin E may be regenerated from its reduced form by ascorbate, which has an important antioxidant function in maintaining the pool of membrane-located vitamin E. Vitamin C and vitamin precursors (*e.g.*, carotenoids) may also act to reduce the rate of initiation or prevent the propagation of free radicals (Kitts, 1997; Halliwell *et al.*, 1995). The importance of vitamins as antioxidants has been discussed more fully in 1.11.5.

In addition to the non-enzymatic antioxidants, cells also contain antioxidant enzymes which remove both radicals and their associated non-radical oxygen species (*i.e.*, hydrogen peroxide). Superoxide dismutase (SOD), catalyses the conversion of superoxide ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) (Halliwell, 1982). Whilst glutathione peroxidase (GSH-Px) metabolises H_2O_2 to H_2O in the cell cytosol and endoplasmic reticulum (Benson, 1990). Metabolising H_2O_2 reduces the possibility of it proving toxic to the cell and prevents its involvement in Haber-Weiss/Fenton chemistry. Furthermore, the reaction of $O_2^{\cdot-}$ and $^{\cdot}OH$ and the reaction of $O_2^{\cdot-}$ with H_2O_2 or lipids may in turn promote the formation of singlet oxygen $^1O_2$, which may be involved in lipid peroxidation and free radical formation (Benson & Noronha-Dutra, 1988). Reduced glutathione (GSH) serves, in part, to set the redox status in tissues (Parihar *et al.*, 1997). GSH confers protection by providing a preferential substrate for S-H oxidation with the production of GSSG (oxidised glutathione) and water (Halliwell, 1982; Scott, 1997).

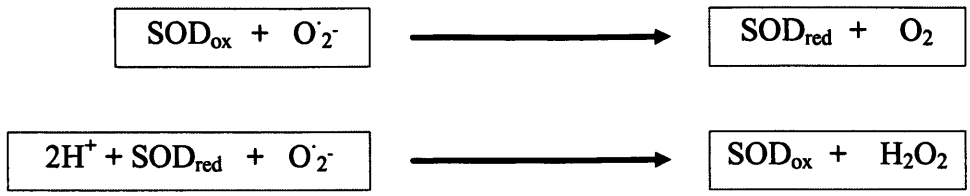
GSH (in conjunction with ascorbate recycling), catalase and several different peroxidases all catalyse the breakdown of H_2O_2 and the oxidation of hydrogen donors (Benson, 1990; Halliwell, 1982). Peroxidases have several other functions in plant tissues including the degradation of indoleacetic acid (and are therefore important in the regulation of plant growth) and the promotion of lignification reactions (Halliwell, 1982; Omran, 1980).

Molecular studies have previously indicated that SOD in plants may be directly induced by oxidative stress (Tsang *et al.*, 1991) and increased SOD activity has been observed in response to stresses including: drought, chilling, anoxia and pathogenic injury (Monk *et al.*, 1989). Studies have also indicated that a link may be demonstrated between the ability of a cell line to survive cryopreservation and its morphogenetic potential (Benson *et al.*, 1992b). Cell lines with a greater capacity for protection against oxidative stress are more readily able to survive cryopreservation than cell lines which have diminished capacity for protection against oxidative stresses induced by freezing (Benson *et al.*, 1992b).

It may therefore be concluded that the effectiveness of antioxidant protection is dependant upon the interaction of several antioxidants, located in different cellular compartments (Benson *et al.*, 1990; Shigeoka *et al.*, 1980). In rice cells reduced catalase and peroxidase activity may be correlated with increased oxidative stress (Benson *et al.*, 1992a,b). The complex interactions between antioxidants and mechanisms for the control of potentially injurious oxidative species derived during periods of stress/damage are also likely to be of importance in the cryopreservation of the algae. As an indication of the antioxidant status of algal tissues four interrelated antioxidant enzymes were selected for study, each of which SOD, catalase, peroxidase and glutathione reductase are discussed in detail below (8.1.1.1-8.1.1.5).

8.1.1.1 Superoxide dismutase

Superoxide dismutase (SOD) refers to a collective of three enzymes characterised by different metal moeities (Cu/ZnSOD, MnSOD and FeSOD) (Scott, 1997). The major form of SOD is Cu/ZnSOD, it may be cytosolic as well as being located in chloroplasts (Benson *et al.*, 1992a,b; Halliwell, 1982). MnSOD is located in the mitochondria and FeSOD in prokaryotes and the plastids of some plants (Salin, 1987). The antioxidant enzymes SOD catalyse the conversion of superoxide ($O_2^{\cdot-}$) to H_2O_2 and O_2. The reaction sequence permits the scavenging of reactive oxygen species and the regeneration of the oxidised catalyst.

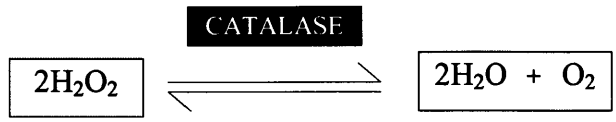


Although SOD effectively removes the potentially injurious $O_2^{\cdot-}$ radical, the reaction product, H_2O_2 is also highly toxic to the cell.

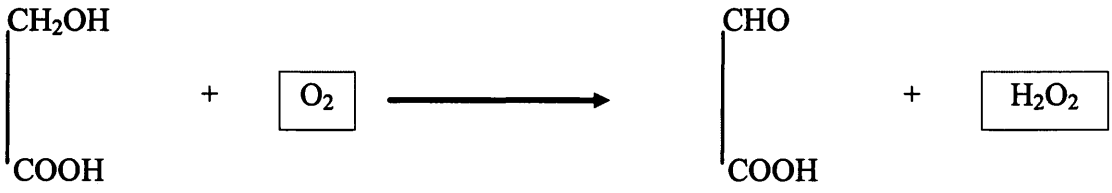
In isolated wheat chloroplasts, monitoring changes in Cu/ZnSOD activity whilst illuminated, demonstrated a reduction in both SOD activity and content with duration of exposure to photooxidative stress, however, addition of ascorbate, a H_2O_2 scavenger, prevented photooxidation-associated inactivation of SOD (Casano *et al.*, 1997). Furthermore, potential inhibition of SOD by H_2O_2, hydrogen peroxide may in the presence of transition metal catalysts, react with $O_2^{\cdot-}$ to form the highly toxic hydroxyl radical ($^{\cdot}OH$) (Chapter 7 and 1.11.2). Cells possess a number of antioxidants which can either directly or indirectly remove H_2O_2, including the antioxidant enzymes catalase, peroxidase and glutathione reductase (Halliwell, 1982; Halliwell, 1974).

8.1.1.2 Catalase

Catalase a haeme-containing antioxidant enzyme, catalyses the breakdown of the potentially damaging H_2O_2 to H_2O and O_2.



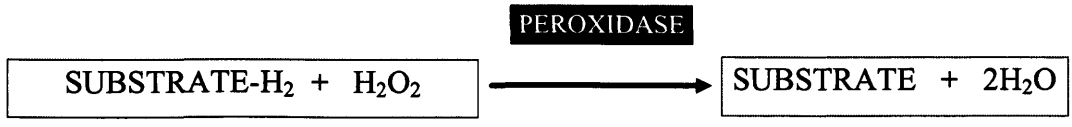
In plants catalase specifically controls H_2O_2 levels in peroxisome metabolism where glycolate oxidase catalyses the O_2 dependant production of glyoxylate from glycolate (Halliwell, 1987).



H_2O_2 produced via the pathway detailed above and SOD activity (8.1.1.1) will become cytotoxic if permitted to accumulate. Toxicity may be due to its involvement in toxic oxidative chain reactions (*e.g.*, Fenton chemistry see 1.11.4) or through its ability to inhibit enzymes (Halliwell, 1987; Halliwell, 1974). Catalase, therefore, has an indirect role in protecting cells from H_2O_2 mediated injury. However, catalase does not dissipate all of the H_2O_2 produced in cells; it is also catalysed by a further system, comprising several different peroxidases (Halliwell, 1982; Halliwell, 1974; Omran, 1980).

8.1.1.3 Peroxidase

Peroxidases are present in comparatively high quantities in higher plants and have a number of specific roles 1.11.5. Several different peroxidases catalyse the H_2O_2 dependant oxidation of substrates (Halliwell, 1982). Peroxidases also have a number of additional functions in plant tissues including: degradation of indoleacetic acid and the promotion of lignification reactions (Halliwell, 1982; Omran, 1980). Measurements of peroxidase activity may also be employed as an indicator of root formation (Jouve *et al.*, 1994; Gaspar *et al.*, 1992; Gaspar, 1990) and may be employed to advance micropropagation procedures from empiricism to propagation techniques developed from knowledge of plant developmental processes (Gaspar, 1990).



Adapted from Halliwell (1982)

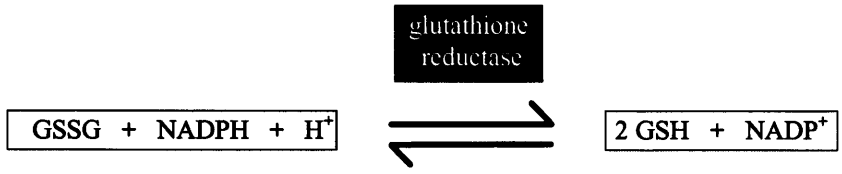
E. gracilis has been reported to contain a peroxidase which specifically requires L-ascorbic acid as the natural electron donor (Shigeoka *et al.*, 1980). Additionally, the system has been suggested to function to remove H_2O_2 in *E. gracilis* (Shigeoka *et al.*, 1980). Furthermore, *E. gracilis* has been reported to lack or only contain very low levels, of catalase compared to higher plants (Brown *et al.*, 1975; Shigeoka *et al.*, 1980). However, Brown *et al.* (1975) also reported that heterotrophically grown *Euglena* may have detectable catalase activity (Brown *et al.*, 1975) and that total levels of peroxidative activity in *E. gracilis* are very low when compared to other algae and higher plants. Ascorbate, glutathione and phospholipase A_2, provide protection from oxidative stress by several routes (8.1.1.4, 8.1.1.5).

In *E. gracilis* the peroxidase, which catalyses the reaction involving ascorbate and H_2O_2 has been described as an unusual haemeprotein stabilised by sucrose and ferrous sulphate (Kow *et al.*, 1982). Kow *et al.* (1982) isolated a NADPH oxidising system, which oxidised NADPH in the presence of ascorbate and H_2O_2 in *E. gracilis*. Light, or dark, grown *E. gracilis* cultures have been reported to contain comparable levels of glutathione (GSH) peroxidase, with the highest levels being detected in cells in midstationary growth phase (Overbaugh & Fall, 1985). The enzyme isolated by Overbaugh & Fall (1985) was Se-independent and was active with H_2O_2 and a variety of organic hydroperoxides.

8.1.1.4 Sulfhydryl groups

Sulfhydryl groups may be divided into non-protein thiols, represented by very low molecular weight compounds [aminoacids (cysteine) or glutathione], or by protein thiols high molecular weight thiols (protein bound sulfhydryls) (Faure & Lafond, 1995) see 1.11.8. Glutathione, a non-protein compound containing a thiol group (S-H), functions by protecting oxygen sensitive enzymes and other proteins from oxidative degradation (Halliwell, 1982). Protection is conferred by providing a preferential substrate for S-H oxidation.

However, one of the products of this reaction oxidised glutathione (GSSG), may, if permitted to accumulate prove toxic to cells (Halliwell, 1982). The enzyme glutathione reductase catalyses its degradation and recycles the protective GSH molecule. Further protective mechanisms involving GSH are discussed in 8.1.1.5.

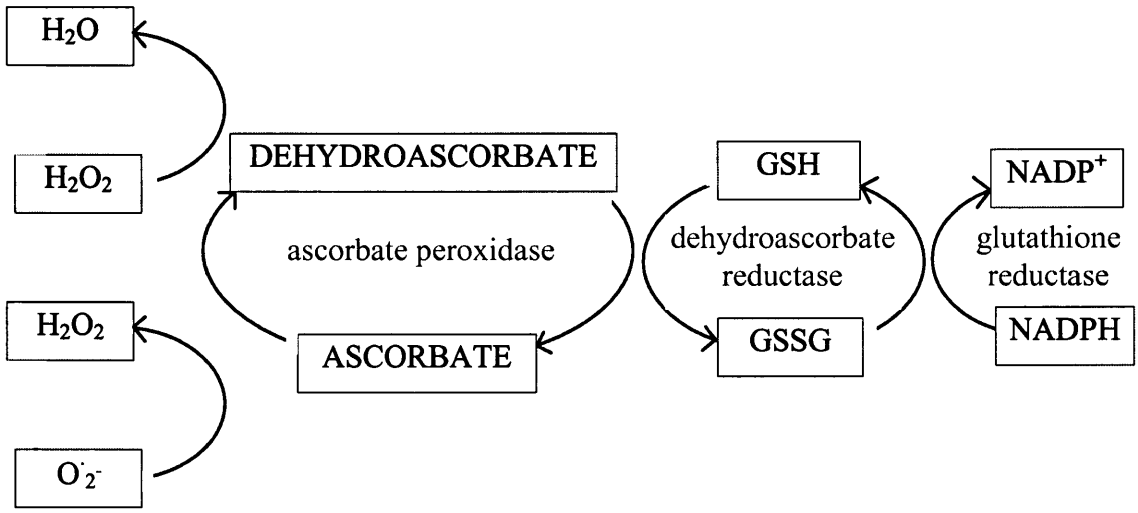


In aquatic animals, SOD catalyses the conversion of superoxide to hydrogen peroxide whilst glutathione peroxidase metabolises the H_2O_2 to H_2O (Parihar *et al.*, 1997). Reduced glutathione (GSH) is an important antioxidant which serves, in part, to set the redox status in tissues. During periods of temperature stress SOD activity increases while GSH-Px activity and GSH content decrease (Parihar *et al.*, 1997).

Protein SH groups have been described as important agents in protecting cells against NO_2 and associated peroxidation of lipids (Halliwell *et al.*, 1992). In addition, the oxidation of protein sulfhydryl (SH) groups has been proposed as a primary reaction leading to membrane damage in plant cells affected by ozone (Chevrier *et al.*, 1988 see 1.11.5.1.) which caused a large decrease in cellular protein SH groups (Chevrier *et al.*, 1988). The oxidation of the sulfhydryl groups is likely to lead to alterations of membrane properties, ionic imbalance and disturbance of metabolic processes which may impair subsequent growth (Chevrier *et al.*, 1988). Preferential oxidation of plasma SH groups (protein SH groups) has been identified as providing protection from nitric oxide and superoxide in blood plasma (Van Der Vliet *et al.*, 1994). In addition, Hu *et al.* (1993) reported that protein SH groups may act as an important protective antioxidant, by acting as a preferential target for attack by hypochlorous acid. Although many examples of antioxidative protection by protein SH groups are from mammalian sources, it would be reasonable to assume that protein SH groups may also function as preferential sites for oxidation in the algae.

8.1.1.5 Glutathione reductase

Glutathione reductase is found in the soluble fraction of both prokaryotic and eukaryotic cells (Goldberg & Spooner, 1983). The primary role of the enzyme is apparently in the maintenance of the intracellular reduced glutathione (GSH) concentration which is required for the reduction of oxidised protein thiol groups (Goldberg & Spooner, 1983). The activity of the enzyme can be assayed spectrophotometrically (Goldberg & Spooner, 1983). Within the chloroplasts GSH functions in conjunction with an ascorbate recycling reaction (Halliwell, 1987). Scavenging of H_2O_2 by ascorbate peroxidase prevents H_2O_2 entering into Haber-Weiss/Fenton reactions which give rise to the highly toxic hydroxyl radical 1.11.2, (Chapter 7).

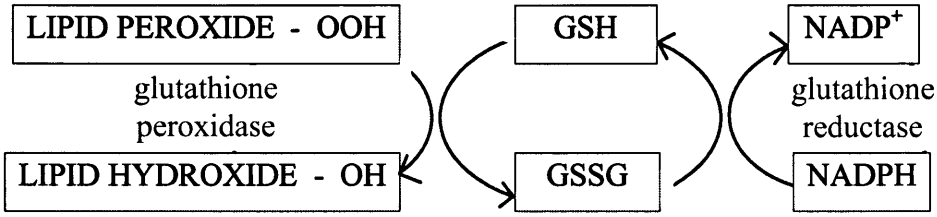


Adapted from Halliwell (1982)

As noted above, GSSG in excessive quantities may prove toxic to cells. The GSSG produced as a consequence of recycling of ascorbate by GSH/dehydroascorbate reductase, which is recycled by the NADPH dependant enzyme glutathione reductase.

GSH serves a third important function in protecting membranes from oxidative damage. As discussed in previous chapters and 1.11.2-1.11.4 unsuccessful quenching of free radicals and toxic oxygen species may promote membrane damage (lipid peroxidation). If these peroxides are not removed, they may induce further free radical chain reactions (Benson, 1990). Lipid peroxidation is a primary route of oxidative stress in biological tissues and has been highlighted as a potential mechanism of cryoinjury in

cryopreserved tissues (Cotterill *et al.,* 1989a). Damage is initiated via a free radical attack on polyunsaturated fatty acids, located in membrane lipids. These reactions form lipid radicals which, upon addition of molecular oxygen, produce lipid peroxyl radicals (Benson *et al.*, 1992a,b). These radicals further react with fatty acids producing a lipid radical and a lipid peroxide, initiating a self propagating chain reaction (Sevanian & Hochstein, 1985).



Adapted from Van Kuik *et al.* (1987)

Lipid peroxides may cause further damage through their breakdown into secondary lipid-oxidation products. In studies performed on model membrane systems, the detoxification of membranes containing phospholipids may occur via a mechanism dependant upon phospholipase A_2, which is involved in increasing substrate availability for GSH by excising the peroxidised lipids from membranes (Van Kuijk *et al.*, 1987), however, this has not been substantiated in higher plants. In addition, the peroxidised lipid is spatially removed from the site of lipid peroxidation chain reactions preventing their further involvement in the initiation of new free radical chain reactions (Van Kuijk *et al.*, 1987). Subsequently, the hydroxy fatty acid produced by GSH peroxidase is reacylated with long chain fatty acyl coenzyme A and reinserted into the damaged membrane (Van Kuijk *et al.*, 1987). These mechanisms are representative of a highly complex repair mechanism.

8.1.2 Antioxidant assays and their application to microalgae

Detailed above are a number of antioxidant enzymes which may prove important in the cryotolerance of algae. In addition, to their presence/absence it is also important to determine antioxidant responses to low temperatures and freezing. In order to achieve this, a series of assays have been selected for the determination of enzyme activity post-

treatment. Many of these antioxidant assays, determine enzyme activity in a supernatant derived from a cellular extraction and have been developed for use in higher plant systems (Benson & Withers, 1987; Benson *et al.*, 1992a,b; Jouve *et al.*, 1993). During the course of this study these techniques have been adapted to study antioxidant activity in unicellular microalgae.

It is vital that the enzyme extraction technique employed permits readily reproducible accurate extraction of the intracellular component of the algae. The extraction technique employed must also guard against exposure of the cells/extract to elevated temperatures which may enhance enzyme breakdown or inactivation due to denaturing of the enzyme itself. Many of the assay techniques employed depend on the extracted enzyme catalysing a compound whilst monitoring the change in absorbance due to the oxidation or reduction of the compound spectroscopically (Benson & Roubelakis-Angelakis, 1992; Benson & Roubelakis-Angelakis, 1994; Aebi, 1983; Murphy & Huerta, 1990; Goldberg & Spooner, 1983). Enzyme activity may then be expressed as a specific activity on the basis of total soluble protein.

The sulphide-disulphide theory of freezing damage was proposed by Levitt in 1962. It was proposed that the freeze-induced destabilisation and oxidation of SH groups in macromolecules would promote denaturation and conformational changes which may impair cellular function (Levitt, 1962). Although freezing injury has since been found to involve many complex events (1.9-1.11.9), SH groups have been shown to be susceptible to freezing in plants and assaying to determine fluctuations in SH content during freeze/thaw cycles and recovery may be useful in algae (Harding & Benson, 1995; Benson & Roubelakis-Angelakis, 1994). Protein and non-protein bound sulfhydryl group status may be determined on tissue suspensions by determining total and non-protein bound groups by measuring spectroscopically the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) to 2-nitro-5-mercaptobenzoic acid by SH groups (Chevrier *et al.* 1988). The SH group content may then be expressed on a cell basis or a Chl. *a* basis.

8.1.3 Objectives

The objectives of these studies were to evaluate the antioxidant status of two algae (*Euglena gracilis* and *Haematococcus pluvialis*) and relate changes and differences in the antioxidant enzymes SOD, catalase, peroxidase and glutathione reductase to cryotolerance and cryoinjury in these organisms. In addition, fluctuations in antioxidant enzyme activities induced by the treatment steps in a two-step cryopreservation protocol and during the course of a 48 h. recovery period were investigated. A further objective of this study was to monitor changes in total and non-protein bound sulfhydryl group content induced by the treatment steps in a two-step cryopreservation protocol and changes during a 48 h. recovery period. Furthermore, the differences between the two algae were investigated in order to compare the antioxidant status of a cryo-tolerant alga (*Haematococcus pluvialis*) and *Euglena gracilis* which is known to be freeze sensitive.

8.2 Materials and methods

8.2.1 Organisms and culture regimes

Cultures of *Haematococcus pluvialis* Flotow CCAP 34/8 and *Euglena gracilis* Klebs CCAP 1224/5Z were selected for study (2.1). Organism culture regimes and recovery conditions were as described in 2.2-2.3.

8.2.2 Cryopreservation procedures

Cryoprotectant solutions were always added to cell suspensions, to avoid excessive toxic shock. Cryoprotectant chemicals employed were: dimethylsulphoxide (DMSO) and methanol (Sigma, USA). Final concentrations of 5 or 10% (v/v) of cryoprotectant were used throughout, with a 5 or 15 min. exposure period at room temperature (RT/20°C) or at 0°C prior to cooling to subzero temperatures.

Vials containing 0.5ml of algae/cryoprotectant were frozen using controlled cooling (see 2.4). Vials containing cells for controlled cooling were cooled to their intermediate holding temperature using a Planer Kryo 10 programmable freezer (Planer, UK),

employing cooling rates of -1°C $min.^{-1}$ and -0.5°C $min.^{-1}$. Vials were cooled to -35°C or -60°C and held at their intermediate temperature for either 15 and 30 min., prior to being plunged directly into LN.

Vials were thawed using a two-step protocol in which vials were first allowed to slowly warm while being held in the air for 1 min., followed by more rapid warming by direct immersion in a pre-heated water bath at 40°C. All vials were agitated until the last ice crystals had melted. The thawed material was then aseptically transferred to appropriate fresh, sterile, medium (2.2) and used in the viability assays as outlined in 2.5. All errors are expressed as standard errors of mean.

8.2.3 Extraction techniques

8.2.3.1 Cell preparation for extraction

Potassium phosphate (50mM) extraction buffer (pH 7) was prepared with KH_2PO_4 (potassium di-hydrogen orthophosphate) and K_2HPO_4 (di-potassium hydrogen orthophosphate). The extraction buffer was supplemented with 1mM $CaCl_2$, 1mM KCl and 1mM EDTA and adjusted to pH 7.

All cryotreatment steps were carried out in minimal JM media to avoid false positive readings due to media. Cells were centrifuged at 2000rpm post-treatment (cryo-treatment step) and the supernatant removed and then re-suspended in 1ml of 0.05M potassium phosphate extraction buffer pH 7 (1 ml). This procedure was repeated twice to ensure all cryoprotectant and medium were removed. A range of approaches were employed for the extraction of cellular material these are detailed in 8.2.3.2-8.2.3.5.

8.2.3.2 Use of sonication for extraction

Cells were transferred to stainless steel centrifuge tubes and flash frozen in LN. Sonication was employed in an attempt to rupture cell membranes. Throughout the sonication procedure care was taken to record any temperature rises. The frozen cells were sonicated with either a sonic probe or a sonic bath, at pre-selected frequencies. For

H. pluvialis and *E. gracilis* the full range of available frequencies were employed for 1 min., 5 min., 15 min. and 20 min. exposure durations. Post-sonication cells were observed using compound microscopy to assess the degree of cell/membrane disruption (2.6.1).

8.2.3.3 Use of vortexing for extraction

Cells were transferred to a centrifuge tube and vortexed in the presence of sand and/or glass beads. Cells were vortexed, at full power for pre-selected periods of 1 min., 5 min., 15 min. and 20 min. Following vortexing cells were observed using compound microscopy to assess the degree of cell/membrane disruption (2.6.1).

8.2.3.4 Use of grinding for extraction

Cell suspension was transferred to a glass mortar and ground with a glass pestle in the presence of sand and 1ml of extraction buffer (8.2.3.1). Cells were ground for pre-selected periods of 1 min., 5 min., 15 min. and 20 min. Following grinding cells were observed using compound microscopy to assess the degree of cell/membrane disruption (2.6.1).

8.2.3.5 Use of lyophilisation for extraction

Cells in extraction buffer were sealed in 2ml Eppendorf tubes and flash frozen in LN. The Eppendorf tubes were then transferred, in a Dewar containing LN, to a pre-cooled freeze-dryer (Edwards, UK). The tube lids were opened and the cells freeze-dried. Immediately after freeze-drying the Eppendorf tubes were resealed and stored under LN.

To extract cellular material, Eppendorf tubes containing the frozen, freeze-dried cellular material were retrieved from the cryostat and transferred to a LN Dewar containing a custom produced brass Eppendorf tube holder, pre-cooled to -196°C. The LN level was maintained at just above the surface of the brass block at all times. The freeze-dried material was ground with an adapted drill fitted with a grinding bit (polypropylene pellet pestle, Sigma, USA). The grinding bits were pre-cooled in LN prior to use and all

material was ground for 5 min. at a fixed, low revolution, grinding speed. Care was taken to ensure that all implements used were pre-cooled in LN prior to being brought into contact with the freeze dried material.

Following grinding the material was re-suspended in 2 ml of chilled extraction buffer (8.2.3.1) and vortexed for 1 min. The vortexed material was then centrifuged in a cold room (4°C) using a pre-cooled micro-centrifuge (4°C). The supernatant was then transferred to a fresh cryovial (1.8 ml) and stored under LN until required. To recover cell extracts for assaying, vials were removed from the LN storage unit and thawed over ice. Material was maintained in ice and the appropriate volume of extract removed for assaying. Immediately after removal of cell extract for assaying, the unused portion of cell extract was refrozen in LN and returned to the LN storage Dewar.

8.2.4 Protein assay

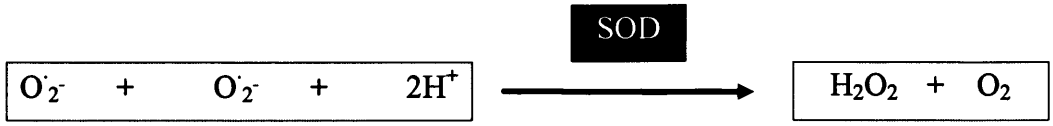
Total soluble protein (see 8.2.3.5) was evaluated using a protein assay kit (Pierce, USA). Assays were performed following the manufacturers guidelines. For all assays, a 0.2 ml sample/standard was employed per 1ml of reagent. Assays were performed in triplicate. Validation runs were performed against the extraction buffer (8.2.3.1), JM media and cryoprotectants to confirm that there was no interference due to these exogenous chemicals.

Protein standards were prepared using the protein standard supplied with the kit (Pierce, USA) and were prepared to cover the range of 5-100 μg protein. Liner regressions were performed on standard curves using Sigma Plot v.3.0 (Jandel Scientific, Germany) at the 95% confidence interval. The constants obtained were employed to mathematically convert ($y = a.x + b$) experimental readings to μg protein.

8.2.5 Superoxide dismutase assay

Superoxide dismutase activity was determined in the supernatant, after extraction by lyophilisation (see 8.2.3.5) (Benson & Roubelakis-Angelakis, 1992; Benson &

Roubelakis-Angelakis, 1994). SOD catalyses the dismutation of the superoxide radical, producing hydrogen peroxide and oxygen.



SOD activity was determined at 25°C, as the inhibition of nitrotetrazolium blue (NBT) reduction by superoxide radicals (produced by light mediated generation from riboflavin and methionine). Four stock solutions (A-D) were prepared in advance (Table 8.1). Stocks A-C were protected from light and all stock solutions were stored refrigerated.

Table 8.1 SOD stock solutions

Stock No.	Compound	Quantity
A)	Riboflavin	4 mg/ 100 ml dH_2O
B)	NBT	40.9 mg/ 50 ml dH_2O
C)	Methionine	0.746 g/ 50 ml dH_2O
D)	"Wing Buffer" pH 7.8	3.40 g KH_2PO_4/500 ml dH_2O
	containing 1×10^{-4} M EDTA[a]	4.36 g K_2HPO_4/500 ml dH_2O

[a] 6.72mg EDTA/200 ml "Wing Buffer"

A reaction cocktail of the stock solutions was prepared, in sufficient quantity to permit all samples and replicates to be assayed using the same batch, immediately prior to assaying and protected from the light. Assay of each sample required: 0.3 ml "stock A" riboflavin, 0.10 ml "stock B" NBT, 0.15 ml "stock C" methionine, 1.00 ml "stock D" "Wing Buffer".

The assay reagents (2.98 ml) were then added to 0.02 ml of the cell extract (previously dispensed into clean identical test-tubes). Control tubes (lacking enzyme) were evenly distributed throughout the rack and all samples were evenly illuminated for 20 mins.

The absorbance at 570 nm was then measured and calculated as change in absorbance at 570nm (Beauchamp & Fridovich, 1971). Experiments were performed in triplicate. Validation runs were performed against the extraction buffer (8.2.3.1), JM media and the cryoprotectants to confirm that no interference due to these exogenous materials was occurring. Enzyme activity were expressed on the basis of total soluble protein and was the mean of three replicate samples.

8.2.6 Catalase (UV, spectrophotometer) assay

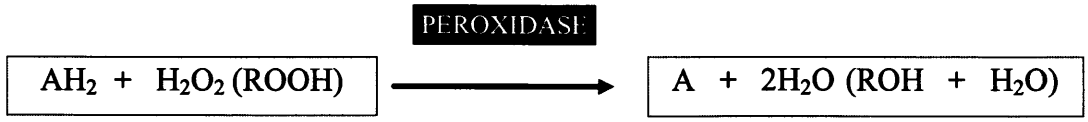
Catalase activity was determined spectroscopically, after extraction by lyophilisation (8.2.3.5) (Benson & Roubelakis-Angelakis, 1994), as the rate of decrease in ultraviolet (UV) absorbance of hydrogen peroxide (H_2O_2) at 240 nm (Aebi, 1983). All assays were performed at 25°C.

Potassium phosphate buffer [(50mM) pH 7] was prepared in advance with KH_2PO_4 and K_2HPO_4 and adjusted to pH 7. The reaction substrate was prepared fresh by diluting 1ml of hydrogen peroxide (H_2O_2) to 400 ml with phosphate buffer. The reaction substrate (2.9 ml) was accurately dispensed into a quartz cuvette, 0.1 ml of sample extract was then added and gently mixed. Samples were then placed in a spectrophotometer, which had previously been zeroed against a "blank" of the phosphate buffer at 240 nm. The spectrophotometer was allowed to settle for 40 s. before recording the absorbance change over 1 min at 240 nm. Validation runs were performed against the extraction buffer (8.2.3.1), JM media and cryoprotectants to confirm that no interference due to these exogenous materials was occurring. Enzyme activity was expressed on the basis of total soluble protein and as the mean of three replicate samples.

8.2.7 Peroxidase assay

Peroxidase activity was measured spectroscopically in the supernatant, after extraction by lyophilisation (8.2.3.5) by assaying for a guaiacol specific peroxidase by measuring

changes in absorbance due to the formation of guaiacol oxidation products at 470 nm (Murphy & Huerta, 1990). All assays were performed at 25°C. The peroxidase enzyme catalyses the oxidation of cellular components by H_2O_2 or organic hydroperoxidases.



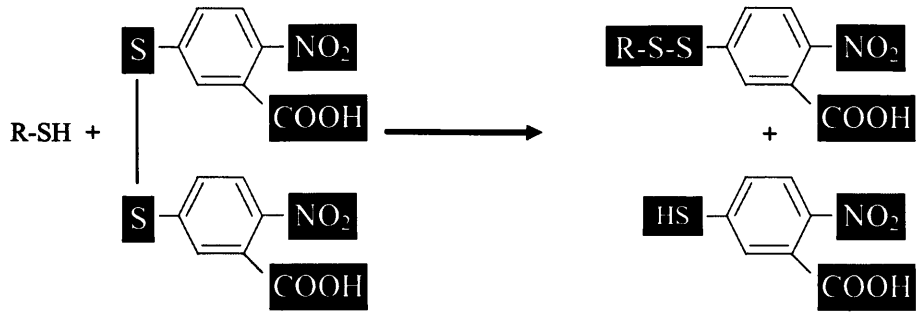
Potassium phosphate buffer, stock solution (50 mM) pH 6.1 was prepared in advance with KH_2PO_4 and K_2HPO_4 and adjusted to pH 6.1.

Using stock potassium phosphate buffer (pH 6.1) a fresh reaction solution of 50 mM phosphate buffer buffer (pH 6.1) with 16 mM guaiacol and 2 mM H_2O_2 was prepared (the 2 mM H_2O_2 was added immediately prior to use).

The sample extract (20 µl) (8.2.3.5) was then added to the reaction solution (980 µl) in a cuvette and gently mixed. The absorbance change over 1 min at 470 nm was recorded using a spectrophotometer previously zeroed against a "blank" of phosphate buffer at 470 nm. Validation runs were performed against the extraction buffer (8.2.3.1), JM media and cryoprotectants to confirm that no interference due to these exogenous chemicals was occurring. Enzyme activity was expressed on the basis of total soluble protein and were the mean of three replicate samples.

8.2.8 Sulfhydryl group assay

Sulfhydryl groups were determined for total and non-protein bound groups as described by Chevrier *et al.* (1988). The determination of SH groups was measured spectroscopically for tissue fractions, based upon the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) by SH groups to 2-nitro-5-mercaptobenzoic acid (see below). The nitromercaptobenzoic acid anion has an intense yellow colour, which is used to determine SH groups (Sedlak & Lindsay, 1968). Assays were performed on cell suspensions, previously obtained from a single cell culture. In addition, cell counts and extraction of Chl. *a* were carried out for cell suspensions as detailed in 2.5.3 and 2.5.6.



Adapted from Sedlak & Lindsay (1968)

Five stock solutions (A-E) were prepared in advance

Table 8.2 SH stock solutions

Stock No.	Concentration	Compound	Quantity	Solvent/volume
A)	10 mM	DTNB	0.3963 g	methanol/100 ml
B)	1.0 M	Tris-HCl pH 8.2	36.33 g	dH_2O/300 ml
C)	0.2 M	Tris-HCl pH 8.5	2.422 g	dH_2O/100 ml
D)	5% (w/v)	SDS		dH_2O
E)	5% (w/v)	TCA		dH_2O

Total SH group levels were determined for cell suspensions (3 ml) by first centrifuging at 2000rpm in order to pellet the cells. The supernatant was then decanted and the cells re-suspended in 2 ml of stock solution "B". This suspension, solutions "D" (0.5 ml) and "A" (0.1 ml) were then added. The cell suspension was then thoroughly vortexed (5 min.) and then incubated at room temperature, with intermittent vortexing (3 × of 30 s. duration) for 20 min. Ice cold methanol (3 ml) was added and the resulting mixture centrifuged for 10 min. The concentration of total SH was determined in the supernatant by measuring A_{412} and comparing to a glutathione standard curve.

Glutathione standards were prepared at 10, 50, 100, 200 and 500 nmol levels. Linear regressions were performed on standard curves using Sigma Plot v.3.0 (Jandel Scientific, Germany) at the 95% confidence level.

Non-protein bound SH groups were determined for cell suspensions (5 ml). The cell suspension was centrifuged to a pellet (as above) and the supernatant decanted. The pelleted cells were re-suspended in 1.5 ml of stock "E", vortexed for 1 min. and then incubated on ice for a further 10 min. and finally was centrifuged 3000rpm for 10 min.

Cuvettes were prepared with 3 ml of stock "C" and 0.1 ml of stock "A". A 1 ml aliquot of the supernatant was then added to the cuvette with gentle mixing and it was incubated at room temperature for a further 2 min. The concentration of non-protein bound SH was determined by measuring A_{412} as above.

Both total and non-protein bound SH group assays were performed in triplicate throughout. Validation runs were performed against JM media and cryoprotectants to confirm that no interference due to any exogenous substances was occurring.

8.2.9 Glutathione reductase assay

Glutathione reductase (GR) activity was determined spectrophotometrically in the supernatant, after extraction by lyophilisation (8.2.3.5), using the method described by Goldberg & Spooner (1983). GR activity was determined through continuous spectrophotometric monitoring of the decrease in absorbance due to the oxidation of nicotinamide-adenine dinucleotide phosphate (NADPH) at 334 nm.

The principle of the assay is represented below, where GSSG and GSH refer to the oxidised and reduced forms of glutathione respectively. Although the reaction is shown as reversible, the reaction forming GSH is strongly favoured (Gloldberg & Spooner, 1983). The catalytic activity of glutathione reductase permits oxidation of NADPH to be monitored by a change in A_{334} as a marker of enzyme activity.

Stock solutions were prepared either in advance (A and B) or immediately prior to the assay (C and D).

Table 8.3 Glutathione reductase stock solutions

Stock No.	Concentration	Compound	Quantity	Solvent/volume
A)	0.12 mol/l	KH_2PO_4	16.33 g	dH_2O/1 l [a]
B)	15 mmol/l	EDTA-$Na_2H_2 \cdot H_2O$	0.56 g	dH_2O/100 ml
C)	65.3 mmol/l	GSSG	40 mg	dH_2O/1 ml [b]
D)	9.6 mmol/l	β-NADPH	8 mg	$NaHCO_3$ solution [1% (w/v)]/1 ml [b]

[a] The phosphate buffer was prepared by dissolving 16.33g of KH_2PO_4 in 800ml of dH_2O, adjusting the pH to 7.2 with NaOH (1 mol/l) then diluting to 1 l with dH_2O.
[b] prepared fresh

Aliquots of stock "A" (2.60 ml) "B" (0.10 ml) and "C" (0.1 ml) were dispensed into a cuvette. An aliquot (0.1 ml) of sample extract was then added to the cuvette and gently mixed. After standing for 5 min. stock solution "D" (0.05 ml) was added. The contents of the cuvette mixture were gently mixed and the change in absorbance at 339 nm over 1 min measured against air. Validation runs were performed against the extraction buffer (8.2.3.1), JM media and cryoprotectants to confirm that no interference due to these exogenous chemicals was occurring. Enzyme activity was expressed on the basis of total soluble protein and were the mean of three replicate samples.

7.2.6 Data analysis using ANOVA

Results were analysed separately using a general linear model of a two way analysis of variance (ANOVA) (Zar, 1996). Data were square root transformed to increase homogeneity of the variances and to increase normality of the residuals. All calculations were performed using Minitab v.11.2 (Minitab, USA).

8.3 Results

8.3.1 Development of extraction techniques

Sonication proved effective in rupturing cell membranes, however, during sonication, large temperature rises were frequently encountered with the cell suspensions being warmed to > 10°C. This indicated that sonication was an unsuitable technique for the extraction of antioxidant enzymes. Vortexing failed to rupture *E. gracilis* or *H. pluvialis* cells and was also unsuitable for the extraction of intracellular material. Grinding in the presence of sand resulted in ruptured cells, however, the technique was extremely labour intensive and microscopical investigations revealed that a large proportion of cells remained intact. In addition, it proved difficult to carry out the entire grinding procedure without exposing the cells to temperature increases as high as 10°C. This made grinding an unsuitable technique for the extraction of antioxidant enzymes.

In contrast, lyophilisation provided a reliable and robust protocol for the extraction of intracellular material. It avoided any loss of enzyme activity due to denaturing at temperatures above 0°C and was employed as the standard method in this study.

8.3.2 Antioxidant assays

Unlike previous chapters where results have been presented separately on each alga, in this chapter, to facilitate interpretation of differences in the endogenous antioxidant levels between *E. gracilis* and *H. pluvialis* and compare variations between the antioxidant responses in the cryo-tolerant alga (*H. pluvialis*) and the freeze sensitive *E. gracilis* the results have been presented on an assay by assay basis.

8.3.2.1 Superoxide dismutase

Significant differences were detected between the algae and treatments when data was analysed using a general linear model of a two way ANOVA. *E. gracilis* was found to have significantly higher level of SOD activity than *H. pluvialis*, in addition, alga and treatment were determined to act synergistically ($F_{6,107} = 6.49$, $P < 0.001$) (Fig 8.1).

Furthermore, exposing *E. gracilis* to treatments in either a non-frozen environment, or in a frozen environment, had a significant affect on the levels of SOD activity ($F_{1,55} = 31.07$, $P < 0.0001$) [Fig 8.1 (a-d, e-g)]. Exposure to a frozen environment significantly increased SOD activity in *E. gracilis*. SOD activity significantly increased during recovery and *E. gracilis* displayed a synergistic interaction between treatment and recovery period ($F_{4,52} = 6.50$, $P < 0.0001$) [Fig.8.1 a-g (dark green, light green, yellow)].

Exposing *H. pluvialis* to treatments in a non-frozen environment or in the frozen environment, also significantly affected levels of SOD activity (Fig. 8.1). These acted synergistically with recovery period, with SOD activity increasing during recovery ($F_{4,51} = 4.86$, $P < 0.002$) [Fig 8.1 a-g (dark blue, light blue, cyan)].

SOD activity increased in all samples exposed to stress in both the frozen and non-frozen environment, the highest SOD activity was in cells exposed to subzero stresses. However, as detailed above, the change in SOD activity in *E. gracilis* after exposure to a frozen environment was significantly higher than that detected in *H. pluvialis* (Fig. 8.1).

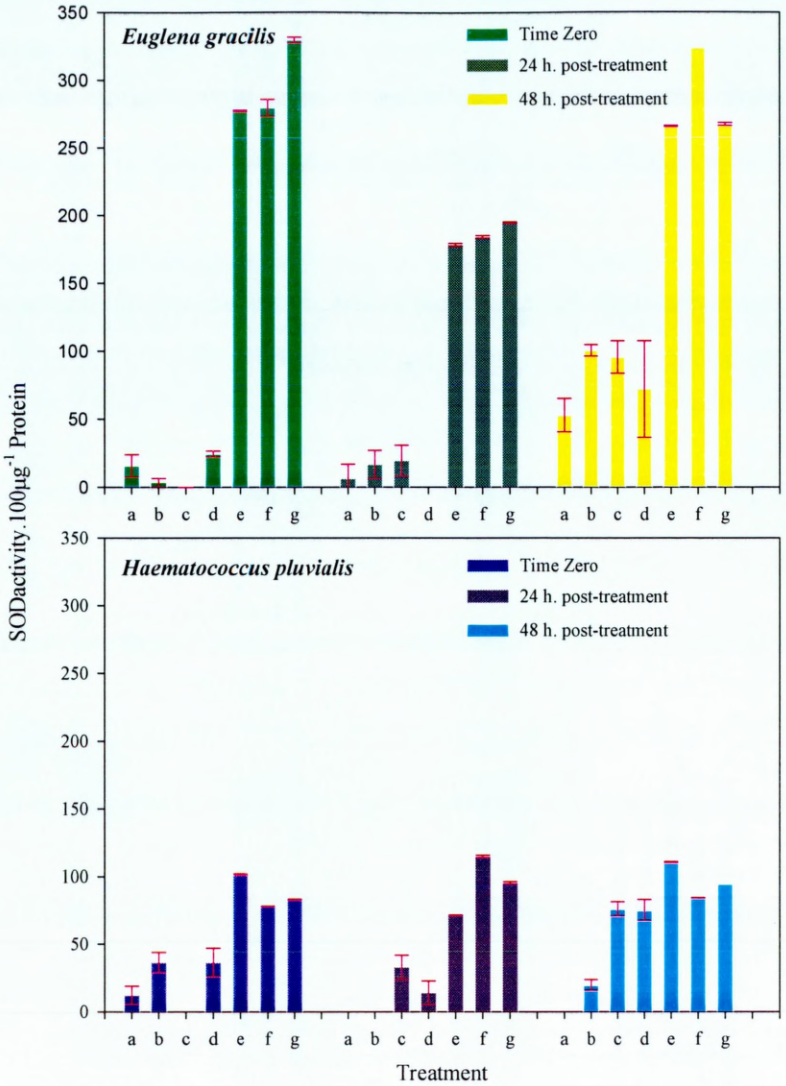


Figure 8.1 Changes in units of SOD activity in *Euglena gracilis* and *Haematococcus pluvialis*.100µg^{-1} protein after exposure to different stages of a cryopreservation protocol.

(a) Untreated control cells (20°C/RT), (b) Cells cooled to 0°C and held for 15 min., (c) Cells exposed to cryoprotectant at RT[a] for 15 min., (d) Cells exposed to cryoprotectant at 0°C[*] for 15 min., (e) Cells control cooled to a predetermined intermediate temperature[#] and held for 30min, (f) Cells plunged into LN from the intermediate holding temperature, (g) Cells plunged directly into LN, without cryoprotectant.

All material was thawed using a simple two-step protocol.

n = 3, errors are expressed as standard errors of mean.

[*] *H. pluvialis* exposed to 5% (v/v) DMSO. *E. gracilis* exposed to 10 % (v/v) methanol.
[#] *H. pluvialis* control cooled from 22°C at -1°C min.$^{-1}$ to -35°C. *E. gracilis* control cooled from 0°C at -0.5°C min.$^{-1}$ to -60°C

8.3.2.2 Catalase

Significant differences were detected for organism and treatment when a general linear model of a two way ANOVA was performed on data which had been square root transformed. *H. pluvialis* had significantly higher level of catalase activity than *E. gracilis*, in addition, organism and treatment were found to act in a synergistic fashion ($F_{6,111}$ = 2.21, P < 0.047) (Fig. 8.2). Catalase activity in *H. pluvialis* was observed to increase during the recovery (Fig. 8.2). In addition, the maximum levels of catalase activity were detected in cells exposed to stresses in the non-frozen environment [Fig 8.2 (a-d), (dark blue, light blue, cyan)]. Although, significant levels of catalase activity were detected in extracts from *H. pluvialis*, catalase activity in *E. gracilis* is considered to be at the limits of detection for the assay (Fig. 8.2). Exposing *H. pluvialis* to treatments in either a non-frozen environment, or in a frozen environment, significantly affected levels of catalase activity and acted synergistically with recovery period ($F_{2,56}$ = 14.20, P < 0.00) [Fig. 8.2 (a-d, e-g)].

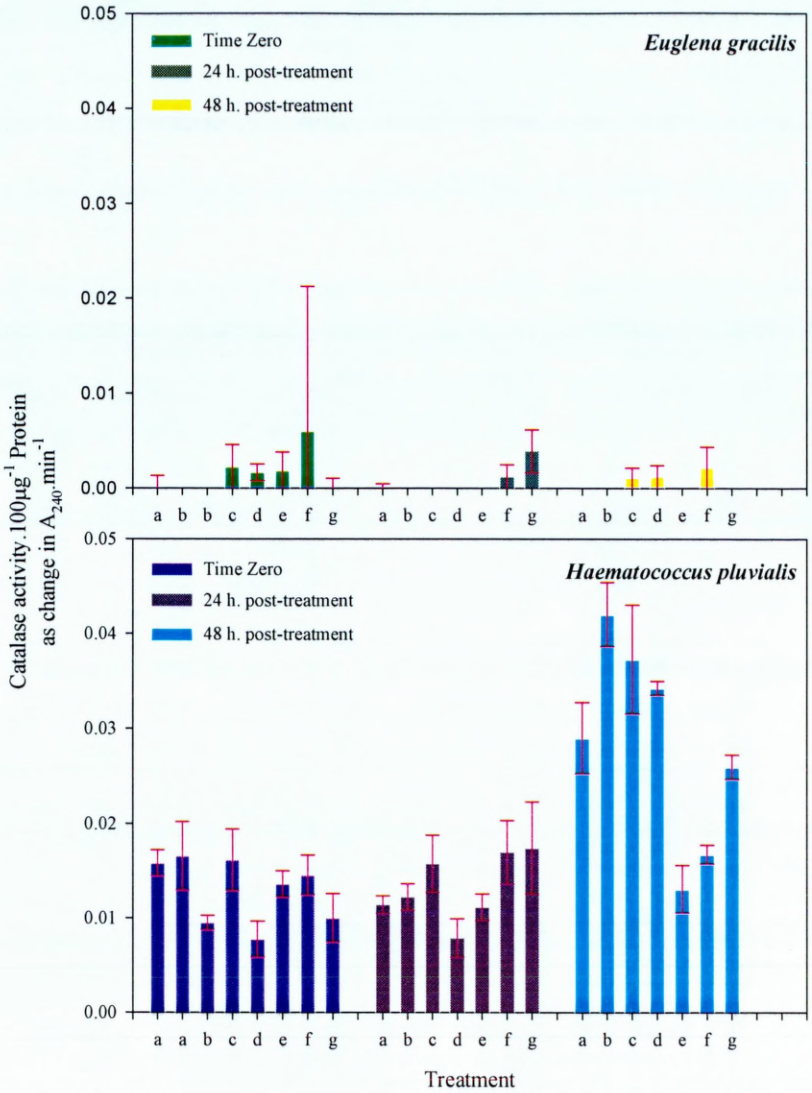


Figure 8.2 Changes in catalase activity in *Euglena gracilis* and *Haematococcus pluvialis* per 100µg.ml^{-1} protein, after exposure to different stages of a cryopreservation protocol.

(a) Untreated control cells (20°C/RT), (b) Cells cooled to 0°C and held for 15 min., (c) Cells exposed to cryoprotectant at RT[a] for 15 min., (d) Cells exposed to cryoprotectant at 0°C[*] for 15 min., (e) Cells control cooled to a predetermined intermediate temperature[#] and held for 30min, (f) Cells plunged into LN from the intermediate holding temperature, (g) Cells plunged directly into LN, without cryoprotectant.

All material was thawed using a simple two-step protocol.

n = 3, errors are expressed as standard errors of mean.

[*] *H. pluvialis* exposed to 5% (v/v) DMSO. *E. gracilis* exposed to 10 % (v/v) methanol.
[#] *H. pluvialis* control cooled from 22°C at -1°C min.$^{-1}$ to -35°C. *E. gracilis* control cooled from 0°C at -0.5°C min.$^{-1}$ to -60°C

8.3.2.3 Peroxidase

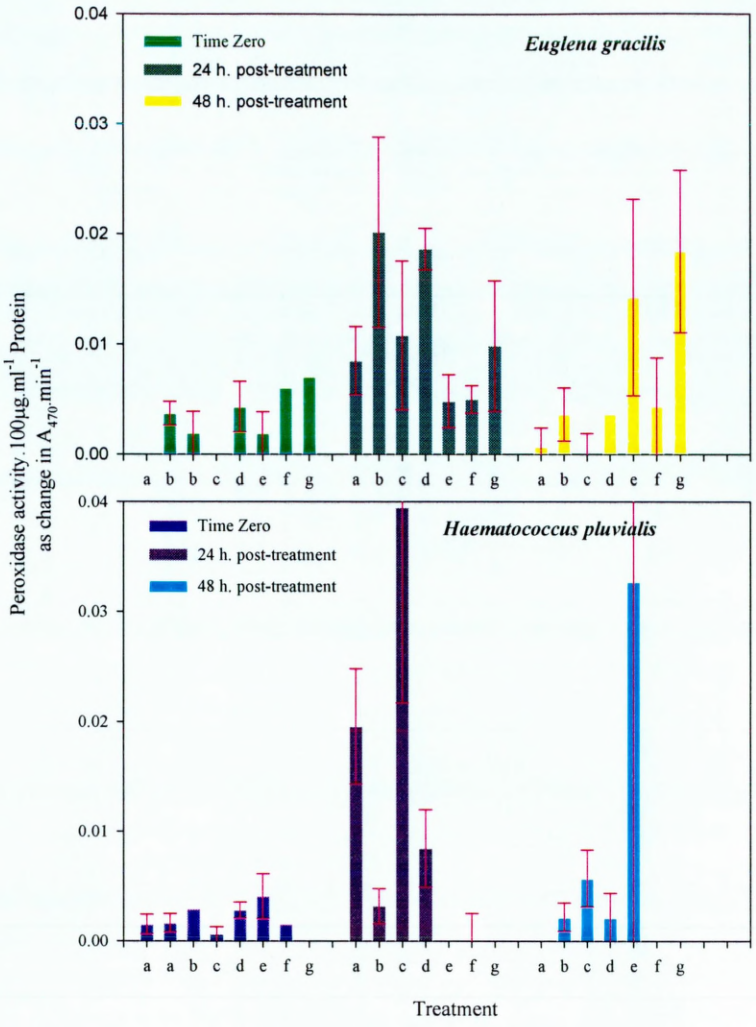


Figure 8.3 Changes in peroxidase activity in *Euglena gracilis* and *Haematococcus pluvialis* per 100µg.ml^{-1} protein, after exposure to different stages of a cryopreservation protocol.

(a) Untreated control cells (20°C/RT), (b) Cells cooled to 0°C and held for 15 min., (c) Cells exposed to cryoprotectant at RT[a] for 15 min., (d) Cells exposed to cryoprotectant at 0°C[*] for 15 min., (e) Cells control cooled to a predetermined intermediate temperature[#] and held for 30min, (f) Cells plunged into LN from the intermediate holding temperature, (g) Cells plunged directly into LN, without cryoprotectant. All material was thawed using a simple two-step protocol. Assay optimised for guaiacol specific peroxidase.

n = 3, errors are expressed as standard errors of mean.

[*] *H. pluvialis* exposed to 5% (v/v) DMSO. *E. gracilis* exposed to 10 % (v/v) methanol.
[#] *H. pluvialis* control cooled from 22°C at -1°C min.$^{-1}$ to -35°C. *E. gracilis* control cooled from 0°C at -0.5°C min.$^{-1}$ to -60°C

Peroxidase activity of "marker" guaiacol specific peroxidase was found to be at the limits of detection for the assaying technique employed for both *E. gracilis* and *H. pluvialis* (Fig. 8.3).

8.3.2.4 Sulfhydryl groups

Significant differences were detected for organism and treatment when a general linear model of a two way ANOVA was performed. *E. gracilis* had significantly higher levels of non-protein SH groups than *H. pluvialis*. In addition, organism and treatment were determined to interact in a synergistic fashion ($F_{6,111} = 5.98$, $P < 0.0001$) (Figs. 8.4, 8.5 8.6.).

Also, exposing the *E. gracilis* to treatments in either a non-frozen environment, or in a frozen environment, significantly affected levels of non-protein SH groups, additionally, there was a significant synergistic interaction with recovery period ($F_{4,53} = 3.75$, $P < 0.009$) [Fig. 8.5 non-protein SH (a-d, e-g)]. Exposure to stresses in the frozen environment resulted in a reduction in non-protein bound SH groups. Non-protein bound SH groups significantly increased during the recovery, furthermore, cryoprotectant exposure, or cooling to subzero temperatures significantly reduced the levels of non-protein bound SH groups in *E. gracilis* and the interaction between recovery and treatment was determined to be synergistic ($F_{12,41} = 7.83$, $P < 0.0001$) [Fig. 8.5 non-protein SH (b-g)]. Exposing *H. pluvialis* to treatments in either a non-frozen environment or a frozen environment did not significantly influenced levels of non-protein SH groups (Fig. 8.6). However, the duration of recovery period did cause a significant increase non-protein bound SH groups ($F_{4,57} = 63.80$, $P < 0.0001$) [Fig. 8.6 non-protein SH (dark green, light green, yellow)]. *E. gracilis*, also had significantly higher levels of protein SH groups than *H. pluvialis* [Figs. 8.4, 8.6 protein SH (a-g)]. In addition, the alga and treatment interacted in a synergistic fashion ($F_{6,109} = 6.13$, $P < 0.001$) [Figs. 8.4, 8.6 protein SH (a-g)]. Furthermore, treatment and recovery had a significant, synergistic interaction on the levels of protein SH groups in *E. gracilis* ($F_{12,40} = 16.49$, $P < 0.0001$) [Fig. 8.4 protein SH (a-g)]. Protein SH groups increased in cultures which had been cooled to subzero temperatures, this increase in protein SH groups appeared to peak 24 h. post-thaw [Fig. 8.4 protein SH (pink, purple)].

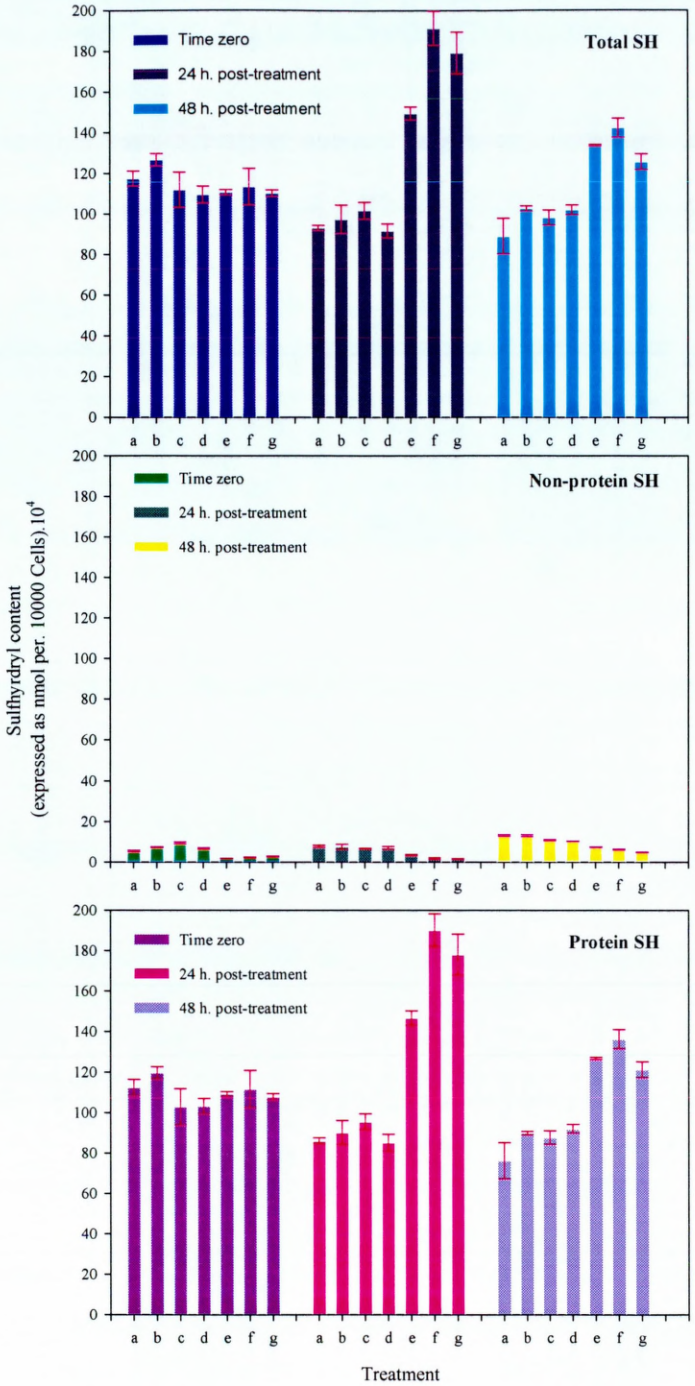


Figure 8.4 Changes in sulfhydryl concentration expressed as total, protein and non-protein sulfhydryl groups in *Euglena gracilis*, immediately post-treatment.

(a) Untreated control cells (20°C/RT), (b) Cells cooled to 0°C and held for 15 min., (c) Cells exposed to cryoprotectant at RT (10% (v/v) methanol) for 15 min., (d) Cells exposed to cryoprotectant at 0°C (10% (v/v) methanol) for 15 min., (e) Cells control cooled from 0°C at -0.5°C $min.^{-1}$ to -60°C and held for 30min, (f) Cells plunged into LN from -60°C, (g) Cells plunged directly into LN, without cryoprotectant.

All material was thawed using a simple two-step protocol.

n = 3, errors are expressed as standard errors of mean.

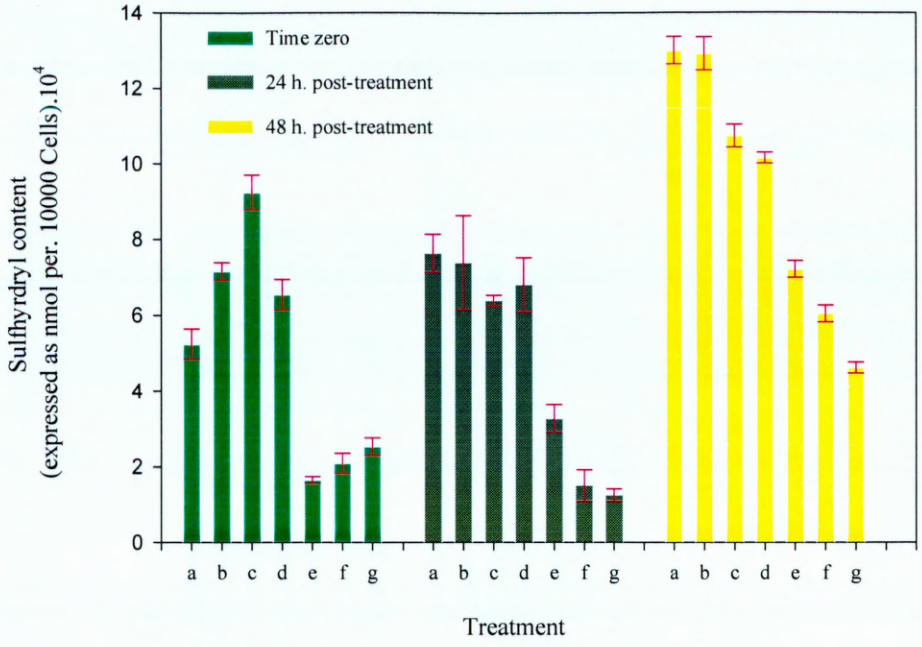


Figure 8.5 Changes in non-protein sulfhydryl group concentration in *Euglena gracilis* after exposure to different stages of a cryopreservation protocol.

(a) Untreated control cells (20°C/RT), (b) Cells cooled to 0°C and held for 15 min., (c) Cells exposed to cryoprotectant at RT (10 % (v/v) methanol) for 15 min., (d) Cells exposed to cryoprotectant at 0°C (10 % (v/v) methanol) for 15 min., (e) Cells control cooled from 0°C at -0.5°C $min.^{-1}$ to -60°C and held for 30min, (f) Cells plunged into LN from -60°C, (g) Cells plunged directly into LN, without cryoprotectant.

All material was thawed using a simple two-step protocol.

n = 3, errors are expressed as standard errors of mean.

A significant synergistic interaction, influencing the levels of protein SH groups, existed between treatment step and recovery period for *H. pluvialis* ($F_{12,41}$ = 4.27, P < 0.000) [Fig 8.6 protein SH (a-g)]. However, the highest protein SH group concentrations were detected in cultures which had not been exposed to cooling to below 0°C, during the recovery protein SH group concentrations were observed to increase [Fig. 8.6 protein SH (pink, purple, a-d)].

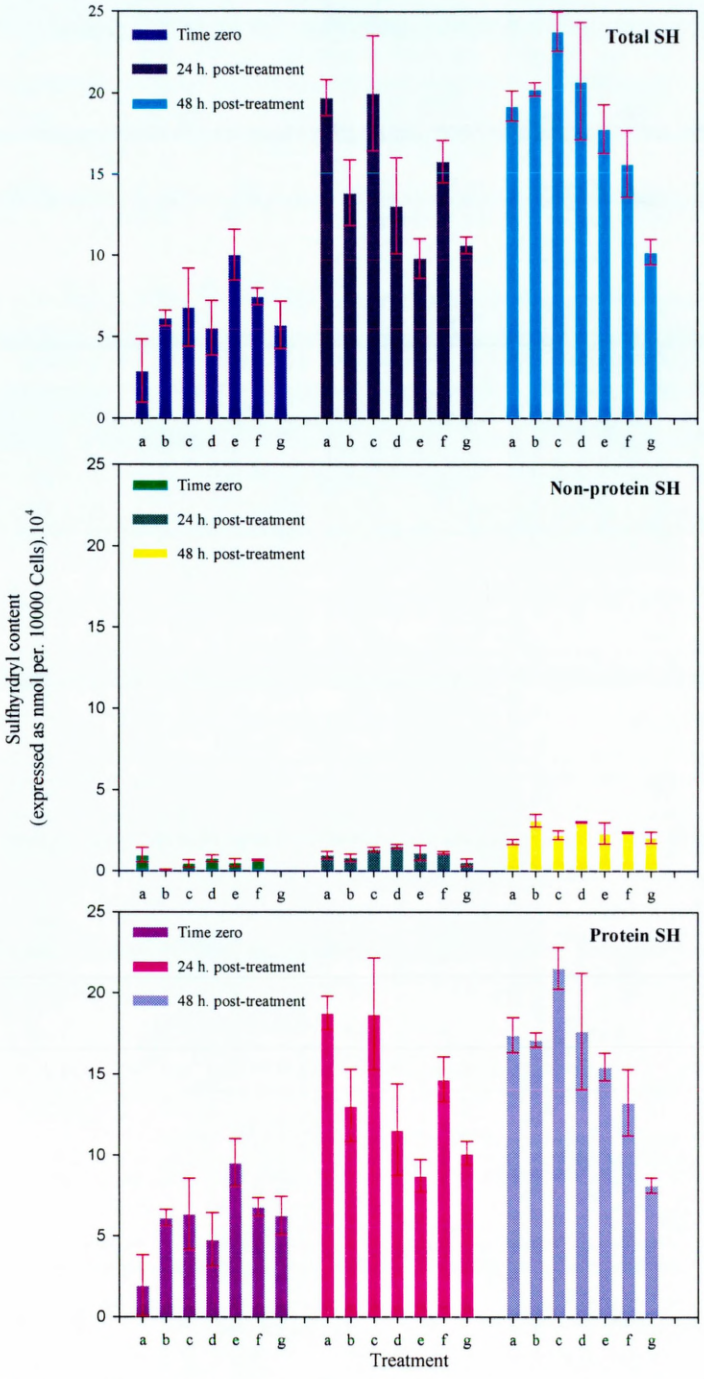


Figure 8.6 Changes in sulfhydryl concentration expressed as total, protein and non-protein sulfhydryl groups in *Haematococcus pluvialis*, immediately post-treatment.

(a) Untreated control cells (20°C/RT), (b) Cells cooled to 0°C and held for 15 min., (c) Cells exposed to cryoprotectant at RT (5% (v/v) DMSO) for 15 min., (d) Cells exposed to cryoprotectant at 22°C (5% (v/v) DMSO) for 15 min., (e) Cells control cooled from 22°C at -1°C $min.^{-1}$ to -35°C and held for 30min, (f) Cells plunged into LN from -35°C, (g) Cells plunged directly into LN, without cryoprotectant.

All material was thawed using a simple two-step protocol.

n = 3, errors are expressed as standard errors of mean.

8.3.2.5 Glutathione reductase

Significant differences were detected for organism and treatment when a general linear model of a two way ANOVA was performed. *E. gracilis* had significantly higher level of glutathione reductase activity than *H. pluvialis* (Fig. 8.7). In addition, a synergistic interaction was detected between organism and treatment ($F_{6,91} = 3.70$, $P < 0.002$) (Fig. 8.7). Furthermore, exposing the algae to treatments in a non-frozen, or a frozen environment had a significantly increased in glutathione reductase activity ($F_{1,101} = 7.37$, $P < 0.008$) (Fig. 8.7). Exposing *H. pluvialis* to treatments in either a non-frozen, or a frozen environment had a significant affect on glutathione reductase activity and interacted synergistically with recovery period, exposure to subzero temperatures increased glutathione reductase activity, in addition, glutathione reductase activity was observed to increase slightly during the recovery ($F_{2,44} = 2.57$, $P < 0.088$) [Fig. 8.7 (dark blue, light blue, cyan)]. A significant effect on glutathione reductase activity was also found in *E. gracilis* due to treatment in either a non-frozen environment or in a frozen environment which interacted synergistically with recovery period ($F_{2,49} = 4.13$, $P < 0.022$) [Fig. 8.7 (dark green, light green, yellow)]. Exposure to subzero temperatures caused an increase in glutathione reductase activity, in addition, glutathione reductase activity increased during the recovery [Fig. 8.7a-g (dark green, light green, yellow)]. However, as detailed above changes in glutathione reductase activity in *E. gracilis* after exposure to a frozen environment were significantly greater than those detected in *H. pluvialis* (Fig. 8.7).

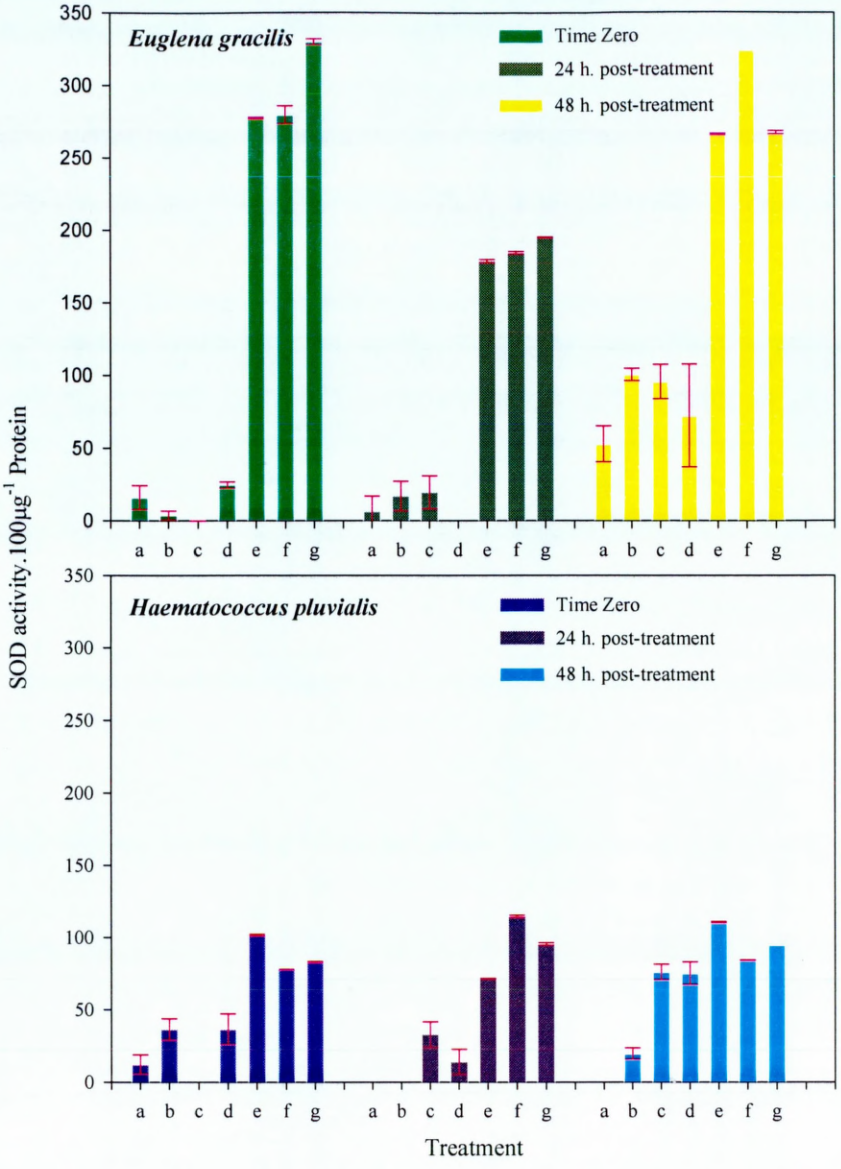


Figure 8.7 Changes in glutathione reductase activity in *Euglena gracilis* and *Haematococcus pluvialis* per 100µg.ml^{-1} protein, after exposure to different stages of a cryopreservation protocol.

(a) Untreated control cells (20°C/RT), (b) Cells cooled to 0°C and held for 15 min., (c) Cells exposed to cryoprotectant at RT[a] for 15 min., (d) Cells exposed to cryoprotectant at 0°C[a] for 15 min., (e) Cells control cooled to a predetermined intermediate temperature[b] and held for 30min, (f) Cells plunged into LN from the intermediate holding temperature, (g) Cells plunged directly into LN, without cryoprotectant.

All material was thawed using a simple two-step protocol.

n = 3, errors are expressed as standard errors of mean.

[a] *H. pluvialis* exposed to 5% (v/v) DMSO. *E. gracilis* exposed to 10 % (v/v) methanol.
[b] *H. pluvialis* control cooled from 22°C at -1°C min.$^{-1}$ to -35°C. *E. gracilis* control cooled from 0°C at -0.5°C min.$^{-1}$ to -60°C

8.4 Discussion

Lyophilisation proved to be a reliable technique for the extraction of antioxidant enzymes from microalgae. Antioxidant assays performed on the cell-free supernatant, obtained using the technique employed indicated that the antioxidant enzymes could be readily and reproducibly extracted from the algae. In addition, no indication of enzyme breakdown or denaturing due to exposure to elevated temperatures was evident.

8.4.1 Antioxidant enzymes

In Chapters 3 and 5 the different responses of *H. pluvialis* and *E. gracilis* to chilling and freezing were highlighted. *H. pluvialis* proved readily amenable to cryopreservation and investigations using an oxygen electrode did not indicate a large inhibitory effect on photosynthetic capacity due to exposure to subzero temperatures. However, *E. gracilis* was observed to experience a large inhibition in photosynthetic capacity after exposure to subzero temperatures. In chloroplasts damaged by low temperatures and freezing, increased H^+ permeability of the thylakoid membrane has been reported with linear photosynthetic electron transport being stimulated whilst the uncoupled electron flow is simultaneously decreased (a partial inhibition of the electron transport chain) (Santarius, 1987). In *E. gracilis*, freezing of thylakoid membranes may have resulted in the inactivation of phosphorylation, inducing oxidative stress/injury in cells due to the production of free radicals, primarily in the chloroplasts (Heber *et al.*, 1971; Santarius, 1987) (see Figs. 3.6, 5.5).

In addition, exposure to low temperatures has also been reported to slow the energy-consuming enzymes in the Calvin-Benson cycle more than the energy-transducing light reactions thus promoting leakage of energy to oxygen and this presents the possibility of H_20_2 production via oxidative reactions (Wise, 1995) (1.11.2). In Chapter 7, oxidative injury in *E. gracilis* was directly investigated and highlighted the possibility of damage due to hydroxyl radicals produced via Haber-Weiss/Fenton chemistry from H_2O_2. The ability of each organism to regulate free radicals and avoid/limit oxidative injury was studied. Fluctuations in antioxidant status could be

induced in each algal culture studied through exposure to cryoprotectant treatment steps, and exposure to subzero temperatures (Figs. 8.1-8.7).

In Chapter 7 it was suggested that elevated levels of H_2O_2 may be present in stressed cells [by the detection of increased levels of CH_4 (due to the scavenging of $^{\cdot}OH$ by DMSO)] (see Fig. 7.4). Following the effective inhibition of $^{\cdot}OH$ production by the iron chelator desferrioxamine it was hypothesised that the primary route for the production of $^{\cdot}OH$ was via the catalysed production of $^{\cdot}OH$ from H_2O_2 (Halliwell, 1982; 1974; 1989) (see Fig. 7.2). Cryoinjury in *E. gracilis* may therefore involve increased superoxide dismutase activity, which could thereby increase the availability of H_2O_2 (8.1.1.1), which could contribute to the generation of $^{\cdot}OH$ and the initiation of subsequent lipid peroxidation (Burdon *et al.*, 1994). Furthermore, "oxidative stress" was identified as an important aspect of chilling injury in *Arabidopsis thaliana*, which may, in its initial stages be exacerbated by increased levels of superoxide dismutase activity (Burdon *et al.*, 1994).

Assays of antioxidant status in the algae *H. pluvialis* and *E. gracilis* confirmed the presence of significant levels of SOD, and glutathione reductase in both organisms (Figs. 8.1, 8.7). Catalase was only present in significant levels in the alga *H. pluvialis*, with catalase activity being at the limits of detection/or apparently absent in *E. gracilis*. Peroxidase activity of "marker" guaiacol specific peroxidase was considered to be at the limits of detection for both organisms (Fig. 8.3). In previous studies on algae, enzymatic extracts from the alga *Acetabularia* were reported to exhibit low levels of guaiacol specific peroxidase activity after a lag period of 1 min. (Driessche *et al.*, 1984), however, glutathione peroxidase has been detected in *Euglena* (Overbaugh & Fall, 1985). In addition, total absence or only low levels catalase activity have been reported in *Euglena* (Brown *et al.*, 1975; Shigeoka *et al.*, 1980). Both organisms demonstrated significant responses in antioxidant enzyme levels (SOD, catalase, glutathione reductase) in cultures which had been plunged directly into LN [Figs. 8.1, 8.2, 8.7 (g)]. This was likely to be due to the continued activity of enzymes even in non-viable cells. Furthermore, injury due to direct plunging into LN although lethal in all cases, would be unlikely to immediately stop all cellular processes and would therefore permit changes in antioxidant status to occur, due to continued enzymatic/chemical activity. This effect

has previously been reported in Chapter 5, where "false positives" due to vital FDA staining could be detected in lethally freeze-damaged cells up to 24 h. post-thaw.

8.4.1.1 Superoxide dismutase activity

Superoxide dismutase was selected for investigation because it directly removes oxygen radicals and is involved in the production of H_2O_2 which was determined in Chapter 7 to be present elevated levels in stressed cells at (1.11.2). There were relatively high levels of SOD activity in both organisms, although the levels of SOD activity were significantly higher in *E. gracilis* than *H. pluvialis* ($F_{6,107} = 6.49$, $P < 0.00$) (Fig 8.1).

In *E. gracilis* a significant increase in SOD activity was detected in cultures which had been exposed to a frozen environment (-60°C, LN), and a synergistic interaction was observed between the treatment and the duration of recovery, with SOD activity increasing throughout the 48 h. post-thaw period ($F_{4,52} = 6.50$, $P < 0.000$) [Fig.8.1 a-g (dark green, light green, yellow)]. Increased SOD activity, as a result of oxidative stress, has been reported in plants exposed to: drought, chilling, anoxia and pathogenic injury (Monk *et al.*, 1989). Additionally, SOD activity in plants may be directly induced by oxidative stress (Tsang *et al.*, 1991). This presents the possibility of a fast response in the activity of this enzyme to oxidative stress and may explain the rapid increase in SOD activity in *E. gracilis* exposed to low temperature stress. This significant increase in SOD activity is likely to promote an increase in H_2O_2, which may prove toxic to the cell at elevated concentrations, or if not removed by the action of peroxidases/catalase it can lead to the production of the highly toxic hydroxyl radical through Haber-Weiss/Fenton chemistry (Chapter 7, 1.11.2). Although SOD functions as an antioxidant removing the potentially damaging superoxide radical, increased SOD activity may enable H_2O_2 to accumulate to potentially harmful concentrations which may ultimately reduce cell viability after exposure to chilling/freezing injury if H_2O_2 is not removed by other antioxidants, such as catalase and peroxidase.

H. pluvialis also had significant levels of SOD activity which increased, in response to oxidative stress (Fig. 8.1). In cultures exposed to freezing injury the levels of SOD activity were much lower than those detected in *E. gracilis* ($F_{4,51} = 4.86$, $P < 0.002$)

[Fig. 8.1 a-g (dark blue, light blue, cyan)]. This would have resulted in lower levels of H_2O_2 produced by SOD activity which, may be more readily regulated by catalase/peroxidase activity within the organism. The significant difference in SOD activity between the two algae may contribute to the ease with which *H. pluvialis* may be frozen and the comparative recalcitrance of *E. gracilis* (Chapters 3-5).

8.4.1.2 Catalase activity

Studies indicated that catalase was either at the limits of detection or absent in the strain of *E. gracilis* studied. This confirms the observations of other workers who have previously reported that *E. gracilis* lacks catalase, an enzyme normally involved in the removal of potentially toxic H_2O_2 from cells (Brown *et al.*, 1975; Shigeoka *et al.*, 1980). However, *E. gracilis* does contain L-ascorbate acid peroxidase, which is thought to protect cells against peroxides generated during photosynthesis (Shigeoka *et al.*, 1980). In addition, *E. gracilis* contains GSH peroxidase, the activity of which is not increased by growth in the light indicating that the enzyme may play a constitutive role in scavenging H_2O_2 in the cytoplasmic compartment in cells incubated in both light and dark (Overbaugh & Fall, 1985). Alternatively, GSH peroxidase may be involved in the removal of lipid hydroperoxides from cellular membranes, similar to the function described for mammalian GSH peroxidases (Wendel, 1980). In *E. gracilis* it has previously been reported that H_2O_2 produced in the mitochondria and chloroplasts immediately diffuses into the cytosol where ascorbate peroxidase is exclusively located in this alga (Ishikawa *et al.*, 1993b). The rapid diffusion of H_2O_2 has been classed as indispensable in enabling *E. gracilis* to regulate intracellular H_2O_2 levels (Ishikawa *et al.*, 1993b).The peroxidase enzymes, ascorbate and GSH peroxidase, therefore function as the main defence in *E. gracilis* against oxidative damage by H_2O_2 and lipid peroxides generated within the energy generating organelles (Ishikawa *et al.*, 1993b). The presence of antioxidants in the cytosol of *E. gracilis* indicates that the cell does have a antioxidant capable of removing H_2O_2 produced through the activity of SOD.

H. pluvialis had significant catalase activity which responded synergistically to treatments in either a non-frozen environment or frozen environment and the 48h. duration of recovery ($F_{2,56}$ = 14.20, $P < 0.001$) [Fig. 8.2 (a-d, e-g)]. Exposure to the

cryoprotectant at RT and in LN caused a small increase in catalase activity in *H. pluvialis*. However, catalase is predominantly found in the peroxisomes and has a low affinity for H_2O_2 (Ishikawa *et al.*, 1993a,b). Stress due to drought and low temperatures may promote the production of the active oxygen species superoxide and singlet oxygen in chloroplasts by photoreduction of oxygen and energy transfer from triplet excited chlorophyll to oxygen, respectively (Smirnoff, 1993). The resulting hydrogen peroxide and hydroxyl radicals (as a result SOD scavenging of superoxide) are reactive and potentially damaging, causing lipid peroxidation and inactivation of enzymes (see 1.11.2, 1.11.4). They are normally scavenged by a range of antioxidants and enzymes which are present in the chloroplast and other subcellular compartments and an increase in SOD activity may occur (Smirnoff, 1993). However, photorespiratory hydrogen peroxide production in peroxisomes decreases (Smirnoff, 1993).

The increase in catalase activity reach a maximum after 48 h. of recovery in cultures which had not been exposed to freezing [Fig 8.2 (a-d), (dark blue, light blue, cyan)]. This may indicate a response to oxidative stress involving the peroxisomes and an increase in photorespiration. Cellular catalase activity in *H. pluvialis* was slow to develop and large elevations in catalase activity were largely confined to cells which had only been exposed to stresses in the non-frozen environment (Fig. 8.2). It may be concluded that changes in catalase activity in this readily cryopreserved alga do not contribute to its ability to recover from exposure to LN. However, the presence of catalase in addition to the peroxidase enzymes associated with H_2O_2 removal may aid this organism in recovering after exposure to LN and the related oxidative stresses.

8.4.1.3 Peroxidase activity

Peroxidase has previously been correlated with developmental changes in plant cell cultures (Gaspar, 1990; Gaspar *et al.*, 1992). Peroxidases may be involved in the production of oxidation products with physiological roles (Gaspar *et al.*, 1992) and the scavenging of H_2O_2 or organic hydroperoxides, products of lipid peroxidation and SOD scavenging of $O_2^{\cdot-}$ (Halliwell, 1982; Benson *et al.*, 1992a,b). The peroxide assay applied was specific for guaiacol peroxidases, which produce oxidation products with physiological functions and have been linked to developmental changes (Halliwell,

1982; Gaspar *et al.*, 1992; Benson *et al.*, 1992b). Their absence, or presence at very low levels, in the algae studied may be due to the specific nature of these guaiacol specific peroxidase (Fig. 8.3). In higher plants they have been linked to embryogenesis and are recognised as "markers of morphogenesis" with important roles in auxin metabolism and cell wall synthesis (Benson *et al.*, 1992b; Halliwell, 1981). This specific peroxidase is therefore not commonly associated with the removal of H_2O_2, in response to stress induced free radical production. In addition, stresses induced by cryoprotectant exposure, chilling or freezing are likely to promote oxidative injury rather than developmental changes in the algae (particularly during the first 48 h. post-exposure). This may explain the absence/low levels of this peroxidase in the algae studied (Fig. 8.3). However, in plant cells a range of peroxidases are present and, in this respect, the algae may benefit from the measurement of ascorbate and glutathione peroxidases. Previous studies have demonstrated ascorbate peroxidase and glutathione peroxidase metabolism of H_2O_2 in *Chlamydomonas reinhardtii* and *Euglena gracilis* (Overbaugh & Fall, 1985; Shigeoka *et al.*, 1980; Takeda *et al.*, 1997). However, fluctuations in the activity of these enzymes induced by cryoinjury/stress have not been documented.

The peroxidases which are involved in the production of oxidation products with physiological roles are often referred to as the guaiacol peroxidases, because guaiacol is employed as an electron donor in their assay. Neither *E. gracilis* nor *H. pluvialis* had large, readily detectable, levels of these guaiacol peroxidases (Fig. 8.3). However, a further group of peroxidases exists in plants which act as antioxidant scavengers, scavenging H_2O_2 or organic hydroperoxides. Although these enzymes were not assayed directly, a number of other studies have reported the presence of GSH and ascorbate acid peroxidase in *E. gracilis* (Ishikawa *et al.*, 1993a; Overbaugh & Fall, 1985; Shigeoka *et al.*, 1980; Brown *et al.*, 1975).

8.4.1.4 Glutathione reductase activity

The presence of glutathione reductase activity in both *E. gracilis* and *H. pluvialis* is likely to function, within the chloroplasts, with an ascorbate recycling reaction (Halliwell, 1987). The scavenging of H_2O_2 by ascorbate peroxidase and its reduction to H_2O prevents it from involvement in Haber-Weiss/Fenton reactions. The action of

glutathione reductase is in the regeneration of GSH from GSSG. *E. gracilis* was demonstrated to have significantly higher level of glutathione reductase activity than *H. pluvialis* ($F_{6,91}$ = 3.70, P < 0.002) (Fig. 8.7). Exposure to stresses due to chilling (0°C), cryoprotectant exposure at RT, and freezing all resulted in significant increases in glutathione activity immediately post-thaw (Fig. 8.7). The variability in the responses to stresses immediately post-exposure may be due to the cell being in a state of flux at this point in the recovery period where the complex interactions between different antioxidant has been disturbed [Fig. 8.7 (light green)]. During subsequent recovery periods glutathione reductase activity significantly increased in those cultures which had been exposed to freezing stresses, and was particularly evident for cells cooled using a two-step protocol and exposed to LN ($F_{1,101}$ = 7.37, P < 0.008) (Fig. 8.7). However, changes in glutathione reductase activity in *E. gracilis* after exposure to a frozen environment were significantly higher than those detected in *H. pluvialis* (Fig. 8.7). In addition, assays indicated that there was less "variability" in the glutathione reductase response in *H. pluvialis* immediately after treatments, instead an increase in glutathione reductase activity was detected in all cultures exposed to stresses, particularly in cultures cooled to subzero temperatures (Fig. 8.7). This may be due to less disruption of the complex balance between different antioxidants in this alga.

In addition to the recycling of GSSG to GSH, glutathione may also function in protecting membranes from oxidative damage where lipid radicals are involved (see 8.1.1.5) (Benson *et al.*, 1992a,b). Lipid peroxidation is the primary route of oxidative stress in biological tissues and has been highlighted as a potential mechanism of cryoinjury in cryopreserved tissues (Cotterill *et al.,* 1989a). Damage is initiated via free radical attack on polyunsaturated fatty acids, located in membrane lipids. These reactions form lipid radicals which, upon addition of molecular oxygen, produce lipid peroxyl radicals (Benson *et al.*, 1992a,b). These radicals further react with fatty acids producing a lipid radical and a lipid peroxide, initiating a self propagating chain reaction (Sevanian & Hochstein, 1985). The increase in glutathione reductase activity is likely to be detected as changes in levels of non-protein bound SH groups, of which GSH is one of the primary classes. In both *H. pluvialis* and *E. gracilis*, glutathione reductase activity in cultures which had been plunged directly into LN, failed to show continued increases in glutathione reductase activity (24 h., 48 h.) after the initial response detected

immediately after thawing [Fig 8.7 (dark green, yellow, dark blue, cyan)]. This provides further evidence of continued enzyme activity in lethally injured cells after thawing. In order to investigate this further, SH status was investigated.

8.4.2 Sulfhydryl (thiol) groups

8.4.2.1 Non-protein bound SH groups

Reduced glutathione (GSH) functions to protect oxygen sensitive enzymes and other proteins from oxidative degradation by providing a preferential substrate for S-H oxidation (Halliwell, 1982).

E. gracilis had a significantly higher level of non-protein SH groups than *H. pluvialis* ($F_{6,111}$ = 5.98, P < 0.000) (Figs. 8.4, 8.5 8.6.). However, exposing the *E. gracilis* to treatments in either a non-frozen environment, or in a frozen environment, significantly affected levels of non-protein SH groups, with non-protein bound SH groups being significantly reduced after exposure to a frozen environment. Additionally, there was a significant synergistic interaction with recovery period and levels of non-protein bound SH groups increasing during the duration of the 48 h. recovery ($F_{4,53}$ = 3.75, P < 0.009) [Fig. 8.5 non-protein SH (a-d, e-g)]. The largest reduction in non-protein SH groups was detected for cultures which had been plunged directly into LN. This was likely to be due to the preferential oxidation of GSH within the cells with only limited, residual, antioxidant enzyme activity present in these lethally injured cells capable of regenerating GSH from GSSG [Fig. 8.4 (g)]. Enzymatic recycling (Halliwell, 1982; Van Kuijk *et al.*, 1987) (due to residual enzyme activity) of non-protein SH groups may explain the increases in non-protein SH levels detected during the 48 h. recovery. However the systems are extremely complex and makes interpretation of the mode of increase of non-protein SH difficult. Clearly this requires further investigation to clarify responses.

H. pluvialis also showed a significant increase non-protein bound SH groups during the 48 h. recovery period, however, exposure to treatments in either a non-frozen environment or a frozen environment did not significantly affect levels of non-protein

SH groups ($F_{4,57}$ = 63.80, P < 0.0001) [Fig. 8.6 non-protein SH (dark green, light green, yellow)]. This may indicate that the thiol groups in *H. pluvialis* are not acting as sites of preferential oxidation. Alternatively, recycling of GSH from GSSG (Halliwell, 1982; Van Kuijk *et al.*, 1987) may be more efficient in *H. pluvialis* than in *E. gracilis*, however this could mot be confirmed without assaying for these specific enzymes. Recycling of GSH may function in conjunction with the ascorbate recycling reaction to remove H_2O_2 and/or in the detoxification of membranes containing peroxidised lipids (Halliwell, 1982; Van Kuijk *et al.*, 1987) and an interesting area for future study would be the potential role of ascorbate peroxidase (Miyake *et al.*, 1991) in the algal cryo-tolerance. Ascorbate peroxidase has previously been detected in *Euglena* and has been linked to the scavenging of hydrogen peroxide (Miyake *et al.*, 1991; Ishikawa *et al.*, 1993a). The increase in levels of non-protein bound SH groups do suggest that *H. pluvialis* was reacting to oxidative stresses induced experimentally by increasing levels of the protective GSH.

In *E. gracilis*, levels of non-protein bound SH groups were demonstrated to fall after exposure to treatments in the frozen environment, indicating that the preferential oxidation of GSH was likely to be occurring. GSH may therefore be assumed to be providing protection to oxygen-sensitive enzymes and other proteins from oxidative degradation (Halliwell, 1982). *E. gracilis* was also able to increase levels of non-protein bound SH groups during the recovery period, suggesting that *E. gracilis* was reacting to oxidative stresses induced experimentally by increasing levels of the protective GSH, which subsequently acted as preferential sites for SH oxidation. However, the oxidation of GSH may result in excessive accumulation of GSSG which may prove toxic to cells. It is possible that the levels of non-protein bound SH groups (GSH) in *E. gracilis* and the significant increase in their concentrations in cultures exposed to low temperature freezing stress/injury may confer protection through protecting oxygen-sensitive enzymes and other proteins from oxidation (8.4.1.4). Protection, may, however, predispose *E. gracilis* to cryoinjury through excessive accumulation of GSSG. *E. gracilis* also demonstrated a significant increase in glutathione reductase activity in cultures exposed to freezing injury (see 8.4.1.4) which supports the former theory. Glutathione reductase also functions in the operation of GSH in conjunction with the ascorbate recycling reaction to remove H_2O_2 and/or in the detoxification of membranes

containing peroxidised lipids (Halliwell, 1982; Van Kuijk *et al.*, 1987; Gillham & Dodge, 1986). The significant increases in both glutathione reductase and non-protein bound SH groups may be a direct response to the equally large elevation in SOD activity detected in cultures exposed to the frozen environment. The increased SOD activity was likely to cause a significant elevation in H_2O_2 within the cell which may promote increased activity in systems which function in the removal of H_2O_2 (Halliwell, 1982). The relative freeze-recalcitrance of *E. gracilis*, may also be, in part, due to the significantly greater changes in antioxidant levels/activity than those observed in *H. pluvialis*. The large changes may create temporary imbalances in the cell permitting excessive accumulation of potentially toxic metabolites such as GSSG, H_2O_2 and peroxidised lipids.

8.4.2.2 Protein bound SH groups

Lipid peroxidation products may react with proteins (Schauenstein *et al.*, 1964; Shires, 1975; Esterbauer *et al.*, 1990). The cytotoxic 4-hydroxy-2-nonenal interacts with GSH and protein thiol groups causing inactivation (Schauenstein *et al.*, 1964; Shires, 1975). In addition, the reaction of lipid peroxidation products with proteins may cause cross linking (Schauenstein *et al.*, 1964; Shires, 1975).

In blood plasma protection from nitric oxide and superoxide may involve the preferential oxidation of plasma SH groups (protein SH groups) (Van Der Vliet *et al.*, 1994). These plasma SH groups have also been reported to be the most important scavenger of hypochlorous acid, a powerful oxidising and chlorinating agent, in blood plasma (Hu *et al.*, 1993). Hu *et al.* (1993) reported that in studies of oxidative damage to protein amino acid residues, only protein SH groups were oxidised, indicating that the protein SH groups were a major target of attack by hypochlorous acid and may function as an important protective antioxidant. Furthermore, the antioxidants ascorbic acid, protein SH groups, uric acid and α-tocopherol have all been described as being important agents in protecting cells against NO_2 and associated peroxidation of lipids (Halliwell *et al.*, 1992). Although these examples of antioxidative protection, due to protein SH groups are from mammalian sources, it would be reasonable to assume that protein SH groups may also function as preferential sites for oxidation in algae. The

elevated levels of protein SH groups in both *H. pluvialis* and *E. gracilis* detected in cultures 24 h. post-treatment indicated that both organisms were able to respond to increased oxidative stress by enhancing the levels of protein SH groups.

Exposure to a frozen environment induced a significant increase in protein SH levels in *E. gracilis* these interacted synergistically with the duration of recovery ($F_{12,40}$ = 16.49, P < 0.000) [Fig. 8.4 protein SH (a-g)]. Protein SH groups peaked in cultures which had been cooled to subzero temperatures after 24 h. of recovery and appeared to be returning to normal after a further 24 h. post-thaw [Fig. 8.4 (pink, purple)]. This response to stress in *E. gracilis* was, however, specific to cultures which had been cooled to subzero temperatures and may represent acclimation to stress resulting in a more rapid turnover of essential protein SH groups (a positive response). In contrast, *H. pluvialis* displayed the highest protein SH group concentrations in cultures which had not been exposed to cooling to below 0°C, with significantly lower levels of protein SH groups in cultures which had been exposed to subzero temperatures [Fig. 8.6 protein SH (pink, purple, a-d)] ($F_{12,41}$ = 4.27, P < 0.000). The lower levels of protein SH groups in *H. pluvialis* cultures exposed to subzero temperatures may be due to preferential oxidation of these groups due to oxidative damage targeting membranes. However, no decrease in non-protein bound SH groups was detected (Fig. 8.5). This may be due to less inhibition of the photosynthetic pathways in *H. pluvialis* resulting in fewer free radicals being produced which can attack soluble non-protein bound GSH. Additionally, the rapid diffusion of H_2O_2 in *E. gracilis* results in GSH functioning as a primary defence against oxidative damage and would be expected to be detected as a significant reduction in non-protein bound SH groups (Fig. 8.5) (Ishikawa *et al.*, 1993a,b).

Euglenoid cells are bounded by the plasmalemma externally and just within it by a proteinaceous layer which is helical in organisation (Fig. 4.1). The pellicle is comprised of up to 80% protein and the remainder of lipids and carbohydrates (Bold & Wynne, 1985). In contrast, algal cell walls mainly consist of acidic polysaccharides, *e.g.*, alginic acid in many marine species, and pectin in fresh-water species and may only contain low levels of protein (Bold & Wynne, 1985; Crist *et al.*, 1994a). A mixed culture of *H. pluvialis* may contain a high proportion of cells which are entering their resting aplanospore stage during which cells will accumulate carotenoids at concentrations

greater than 1% of their dry biomass (Chaumont & Thepenier, 1995). In studies on *Haematococcus pluvialis* cells have been reported to contain (on a dry basis) moisture 9.3%, crude protein 19.2%, crude fat 26.3%, gross energy 24.1 kJ/g and ash 3.9% (Choubert & Heinrich, 1993). These chemical differences between the chlorophyte *H. pluvialis* and the englenoid *E. gracilis* may explain the significantly higher levels of protein SH levels detected in *E. gracilis* compared to *H. pluvialis* ($F_{6,109}$ = 6.13, P < 0.00) [Figs. 8.4, 8.6 protein SH]. In addition, total carotenoid pigments of *H. pluvialis* were determined to be 2.0% ± 0.2% (on a dry basis) (Choubert & Heinrich, 1993) and these high levels of carotenoids may make this alga more cryotolerant (1.5.5).

8.4.3 Conclusions

Exposure to chilling and freezing stresses induced a response in antioxidant status in both organisms. It is possible that increased SOD activity in *E. gracilis* in response to oxidative stress may result in an excessive accumulation of H_2O_2. It may be speculated that this was the primary reason for *E. gracilis* being more difficult to cryopreserve than *H. pluvialis*. In Chapter 7, it was hypothesised that a mode of cryoinjury in *E. gracilis* may involve increased superoxide dismutase activity, which thereby increased the availability of H_2O_2, which contributed to the generation of $^{\cdot}OH$ and the initiation of subsequent lipid peroxidation (Burdon *et al.*, 1994). This has previously been proposed as an important aspect of chilling injury in *Arabidopsis thaliana*, where initial stages were exacerbated by increased levels of superoxide dismutase activity (Burdon *et al.*, 1994). However, *E. gracilis* was also capable of elevating levels of other antioxidants, particularly those which serve to remove H_2O_2. However, these changes in antioxidants may in themselves have increased oxidative injury in *E. gracilis* when they were poorly regulated. Thus, the high levels of carotenoids in *H. pluvialis* may make this alga more cryo-tolerant.

The apparent lack of catalase in *E. gracilis* and the rapid diffusion of H_2O_2 into the cytosol may have made regulation of oxidative stress, in excess of that encountered as a result of essential reactions, difficult. Both organisms would, however, benefit from more antioxidant studies to measure fluctuations in ascorbate and glutathione peroxidases together with further investigations into SOD activity on a Cu/ZnSOD,

MnSOD and FeSOD unit basis. It would also be interesting to compare the antioxidant responses in other algae to chilling and freezing (*Enteromorpha intestinalis* and *Vaucheria sessilis*).

Chapter 9.

General discussion.

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9.1 Empiricism verses understanding

Recently the editor of Cryo-Letters, Felix Franks commented on the quandary of "lots of data - how much information" (Franks, 1998). The accumulation of too much data and not enough information developed thorough the pursuit of fundamental science is as much a problem for Cryobiology as for other scientific disciplines. Frequently, publications focus on the development of "new techniques" based on the empirical modification of existing techniques to provide "enhanced" levels of post-thaw viability rather than the presentation of "truly new" fundamental observations relating to mechanisms of injury and/or recovery. It would be wrong to overtly criticise this empirical approach as it has resulted in the successful development of new protocols and cryopreservation applications. However, this system of investigation becomes limiting when applied to freeze-recalcitrant systems. For this reason this research project, from the onset, was designed to combine investigative, fundamental and empirical approaches, with the ultimate objective of improving cryo-conservation methods for organisms which were difficult to freeze. This discussion aims to overview the findings presented in this thesis in the context of using both empirical and investigative approaches to assist the development of new cryopreservation protocols for freeze-recalcitrant organisms. The importance of interfacing fundamental and applied cryobiology, for technology transfer to "working" culture collections, is also debated.

The development of a scientific discipline from its empirical beginnings may be considered as part of the natural maturation process for any science. For example, in the field of plant tissue culture, the progressive "development out" of empiricism has been discussed by Gaspar (1990). He reflected on the use of micropropagation procedures which were based on the effects of auxins and cytokinins, without sufficient knowledge of the mechanisms involved and their effect on the establishment of the cultivars (Gaspar, 1990). In cryobiology a comparable move away from empiricism is required if those cultures which are the least tolerant of chilling/freezing are to be preserved. The reviews presented in "*Advances in Low Temperature Biology*" volumes 1-3 (Steponkus, 1992; 1993; 1996) and in "*Free Radical Damage in Stored Plant Germplasm*" (Benson, 1990) highlight the evolution of cryobiology from empiricism to a more scientifically

informed approach, to the development of cryopreservation methodologies. This project has endeavoured to develop, from a largely empirically driven Chapter 3, to a more fundamental understanding of the mechanisms which influence algal viability during a cryopreservation freeze/thaw protocol (Chapters 4, 5, 7, 8).

9.2 Mechanisms of cell damage and recovery in cryopreserved algae

Preservation protocols developed for use in culture collections must be robust, and as far as possible, avoid both selection of preservation-tolerant sub-populations and genetic drift (Chapter 1). They must also permit efficient handling and storage of large numbers of cultures (Chapter 1). However, despite the benefits offered by cryopreservation in liquid or vapour phase nitrogen, it is not routinely employed as a preservation technique in many protistan collections. In addition to the requirements of these culture collections, interest continues to be shown in the feasibility of cryopreserving other, microorganisms, plant tissues [for genetic resource conservation (Gonzalez-Benito *et al.*, 1998)], organs and tissues (Fuller, 1987). Recently, the effects of cryopreservation on sperm organelle function and viability (Thomas *et al.*, 1998) and the long-term implications of tissue cryopreservation techniques have attracted most interest (Mitchell *et al.*, 1998). It has also been suggested that recent advances in the cryopreservation of immature human oocytes and ovarian tissue may herald the emergence of a valuable technology (Oktay *et al.*, 1998).

Cryopreservation methodology must be developed/improved to increase levels of post-thaw viability and to cryopreserve presently freeze-recalcitrant organisms. In order to improve current cryopreservation protocols, those components of the cryogenic process which predispose and/or subject cryoprotected/frozen cells to stress must be identified. During the course of this research, novel investigative techniques including: flow cytometry, cryomicroscopy and gas chromatography have enabled sites of cryoinjury and markers of stress to be identified (see Fig. 9.1).

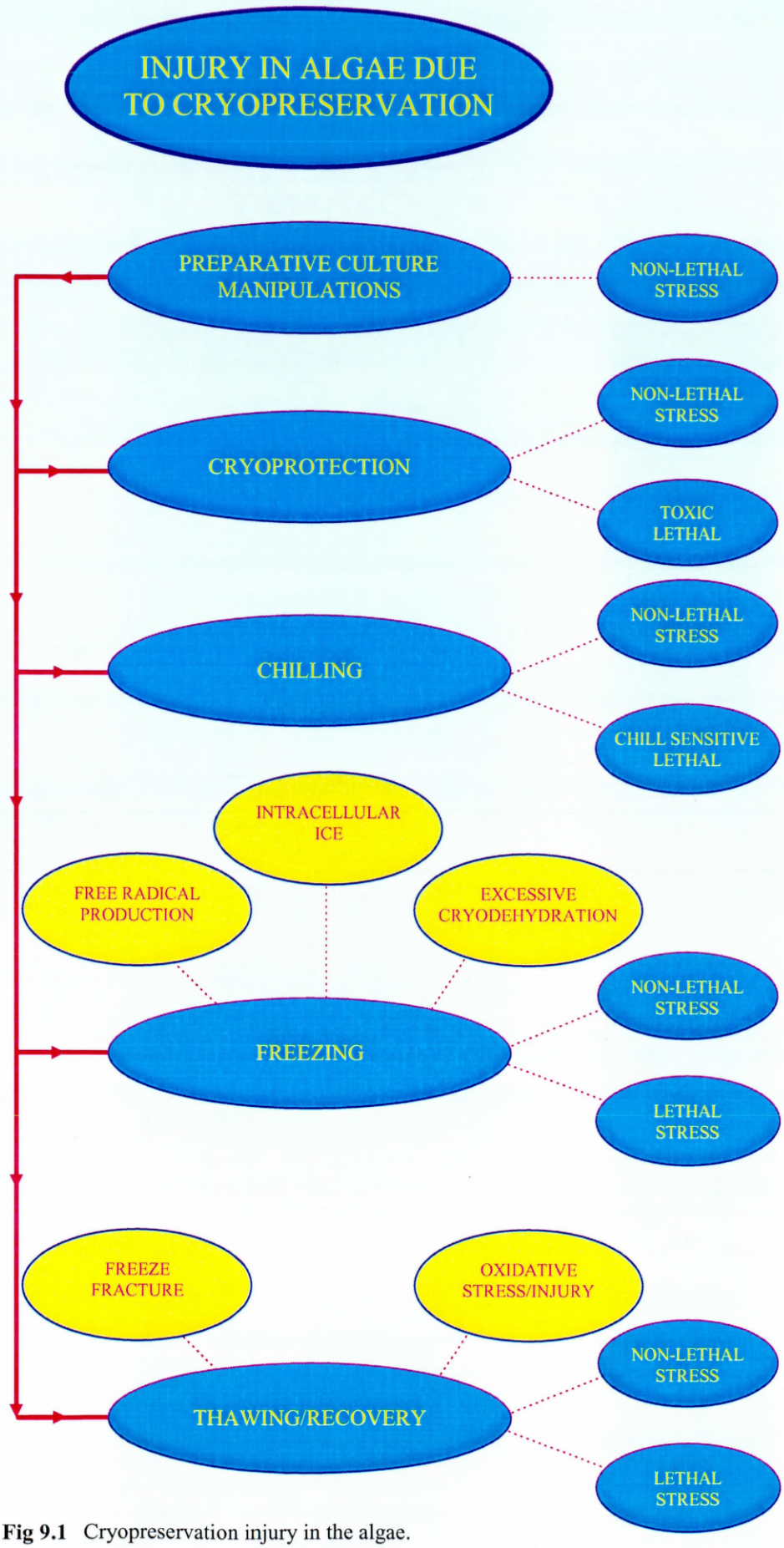

Fig 9.1 Cryopreservation injury in the algae.

Figure 9.1 graphically illustrates a cryopreservation protocol and the stresses which may be associated with each step. In addition, mechanisms which contribute to injury/stress are highlighted for the freezing and thawing/recovery stages of the protocol (Fig. 9.1).

9.2.1 The empirical approach

Chapter 3 described a preliminary investigation of the effects of different two-step cryopreservation protocols on algae. Although this study provided a useful insight into the effects of cryoprotectant exposure, chilling and freezing, little quantifiable or direct evidence was gained regarding the mechanisms of cryoinjury and recovery in the algae examined. However, this approach reflects the primary mode of cryopreservation protocol development employed to date (Chapter 1). Although the successful development of cryopreservation protocols for a diverse range of tissues has been, and continues to be, achieved using this approach (see Chapter 1), more sensitive tissues are likely to remain freeze recalcitrant unless a considered strategic approach to method development is adopted.

9.2.2 The investigative approach

Physical and biological markers of stress and cryoinjury have been identified in this project using flow cytometry and cryomicroscopy and they include: intracellular ice formation (Chapter 4), cryoprotectant toxicity (Chapters 3, 5, 6), osmotic shock (Chapters 4, 5), freeze-fracture events (Chapters 4, 5) and morphological changes (Chapter 4). In addition, markers of free radical attack (Chapter 7) and fluctuations in antioxidant status have been identified (Chapter 8). This has permitted, during the course of this research, a stepwise sequence of stress/injury and antioxidant stress responses to be identified in cultures prepared for cryopreservation (Fig. 9.1) (Chapters 3-8).

Preparative manipulations/cryoprotection (Fig. 9.1): The detection of an accumulation of CH_4 in sealed vials containing *Euglena gracilis* was attributed to elevated $^{\cdot}OH$ levels in the alga (Chapter 7). This provided evidence that stress occurred in the preparative manipulation steps prior to cooling (Chapter 7). The identification of

stress at these points in the cryopreservation protocol highlights the importance of considering all stages in a cryopreservation protocol. However, the contribution of these preliminary preparative steps to an organisms "apparent freeze-recalcitrance" are often discounted by researchers. For example, the common practice of concentrating cultures by centrifugation prior to freezing and/or removal of cryoprotectants by pelleting cells prior to replacing the supernatant with fresh media, has been shown in this study to cause significant stress to algal cells. In this study, the preparative steps were not found to induce lethal stresses, however, it is considered that these stresses may make the organism more sensitive and less likely to overcome additional stresses due to the cryopreservation process. These cumulative stresses may contribute to the freeze-recalcitrance of "more sensitive" organisms (Chapters 5, 7).

Exposure to cryoprotectants has previously been identified as a potentially damaging event, where the toxicity of the cryoprotectant may lethally injure cells (Chapter 1). In this study, sensitivity to cryoprotectants was demonstrated to influence the ability of some algal strains to survive exposure to a frozen environment and in many cases the toxicity of the solutions employed reduced cellular viability (Chapters 3, 6). In cultures exposed to a cryoprotectant solution which had previously been identified as optimum, markers of stress were readily identified including: reduced photosynthetic capacity, increased levels of hydroxyl radicals ($^{\cdot}OH$) and fluctuations in antioxidant levels (Chapters 3, 5, 7, 8). Furthermore, the sensitivity of most of the algae studied to elevated concentrations of cryoprotectants, effectively precluded the development of novel vitrification procedures requiring high molarity vitrification solutions (Chapter 6).

Chilling (Fig. 9.1): Levitt (1980) reported that the sensitivity of many plants to chilling could occur at temperatures as high as 4°C. The potential chilling sensitivity of organisms was discussed in 1.7.1 & 1.7.2, however, none of the algae selected were found to be sensitive to chilling to 0°C. In studies performed on *Euglena gracilis* and *Vaucheria sessilis*, each alga was found to be capable of tolerating chilling to subzero temperatures in supercooled medium without loss of viability (Chapters 4, 5). However, on studies to detect markers of free radical activity, *E. gracilis* cells had increased levels of $^{\cdot}OH$ radicals in cultures chilled to -10°C (Chapter 7). Furthermore, the photosynthetic

capacity of both *E. gracilis* and *V. sessilis* were found to be inhibited by cooling to 0°C (Chapter 5).

Freezing (Fig. 9.1): Excessive cryodehydration, or intracellular ice nucleation, were both identified as causative agents of lethal injury in algae exposed to the frozen environment (Chapters 4, 5). However, in cryomicroscopic studies on fungi neither intracellular ice or hyphal shrinkage were necessarily lethal, but in many cases they affected the rate and quality of growth (Smith & Thomas, 1998). On cooling *Enteromorpha intestinalis* 100% of the thalli survived cryopreservation despite 2% of the cells under going intracellular ice formation (Chapter 4). In this alga, the compartmentalisation of ice nucleation events, where intracellular ice was restricted to individual cells within the multicellular thallus, was believed to contribute to its ability to survive (Chapter 4). In contrast, the coenocytic alga *Vaucheria sessilis* was unable to survive intracellular ice nucleation since its lack of cellular compartmentalisation permitted propagation of intracellular ice throughout the thallus (Chapter 4). This clearly shows the importance of cellular architecture in contributing to freeze tolerance and sensitivity. The potentially lethal effects of excessive cryodehydration and intracellular ice nucleation have both previously been identified in other systems (1.7.5-1.7.6). Furthermore, on freezing algae using "optimum protocols" markers of stress including inhibition of photosynthetic oxygen evolution, increased levels of $^{\cdot}OH$ radicals and fluctuations in antioxidant levels were identified in cells which had been exposed to the frozen environment. "Markers" of stress were also identified where intracellular ice nucleation, or excessive cryodehydration, were unlikely (Chapters 3, 5, 7, 8).

Thawing/recovery (Fig. 9.1): In addition, to damage observed during the preparative steps and cooling, further injuries were attributed to thawing and recovery. As mentioned previously, centrifugation (either during preparative manipulations, or to facilitate cryoprotectant removal) was demonstrated to induce non-lethal stress in *E. gracilis* (Chapters 5, 7). Furthermore, the thawing protocols adopted were demonstrated to affect post-thaw viability levels. To obtain high post-thaw viability levels in *E. gracilis* it was necessary to employ a two-step warming protocol which enabled the intracellular vitreous state to relax prior to rapid warming to room temperature, avoiding glass fracturing and intracellular ice crystal growth (Chapter 5).

The factors contributing to fracture events within frozen systems and ice crystal growth were discussed in 1.9.2. The application of a novel encapsulation/two step cooling protocol was demonstrated to result in high levels of post-thaw viability of cryopreserved of *E. gracilis* (Chapter 6). Protection was considered to be primarily due to alginate providing support to the cells in the frozen environment and thus protecting the cells from freeze fracture events.

9.2.2 Elucidating specific sites of cryoinjury

Membranes (Fig. 9.1): In Chapter 1, the hypothesis of freezing injury may be primarily due to damage to membranes was discussed 1.11.1. Injury was attributed to lysis of the cell/protoplast, leakage of electrolytes and other cell constituents and the breakdown of fine structure (Singh & Miller, 1985). The concept of membrane damage discussed in 1.11.1, was based on observations of the reduction in cell volumes during plasmolysis (Williams *et al.*, 1981; Williams and Hope, 1981) and the "rolling up" and fusing of membranes (Steponkus *et al.*, 1993; Singh & Miller, 1985; Wiest & Steponkus, 1978). Damage in the frozen environment may also be attributed to the degradation of freeze sensitive proteins by the activity of one or several proteases and other degradative enzymes (Uemura & Yoshida, 1986; Stout *et al.*, 1980; Rajasheker *et al.*, 1979, Yoshida, 1979a,b). Ultrastructural effects of low temperature (10°C, chilling) can be observed in membranes and they have been reported to have a profound effect on cellular structure (Kovacs, 1997). Including: swelling of mitochondria and disorganisation of membranes.

Visual markers of membrane stress and damage including flagellar loss were employed to investigate cryoinjury in *E. gracilis* on cooling. In the frozen environment this may possibly cause membrane stress (Chapter 4). TEM ultrastructural studies also indicated increased vesiculation in cells exposed to LN possibly indicating disruption of intracellular membranes (Chapter 4). Membrane rupture was directly observed in *V. sessilis* (Chapter 4). In this alga ultrastructural studies demonstrated massive disruption of subcellular organelles including: extensive damage to mitochondria, endoplasmic reticulum nuclei, distortion of chloroplasts into irregular forms, thylakoid re-

arrangements associated with loss of their stacked, parallel-layered structure and an increase in inner thylakoid spaces (Fig. 4.10) (Chapter 4).

Changes in membranes observed using electron-microscopy, indicated that it is likely that they may also undergo free radical attack. As a result of this the products of lipid peroxidation were monitored (Chapter 7). In TBARS assays performed on *V. sessilis* significant levels of MDA were detected in filaments which had undergone membrane damage due to sectioning, stress caused by cryoprotectant exposure and exposure to a frozen environment (Chapter 7). These results confirm the sensitivity of membranes in this alga to both cryopreservation and pre-cryopreservation manipulations. This provided an initial indication that free radical damage may be involved in algal cryoinjury.

Chloroplasts/thylakoids (Fig. 9.1): In photosynthetic organism's, chloroplasts, and in particular thylakoid membranes, have been identified as being highly susceptible to cryoinjury (Santarius, 1987; 1990a,b). Increased H^+ permeability of the thylakoid membrane in chloroplasts damaged by low temperatures and freezing, has been reported to cause a partial inhibition of the electron transport chain, which may in turn promote oxidative stress (Santarius, 1987). In algae, freezing of thylakoid membranes may therefore result in the inactivation of phosphorylation, inducing oxidative stress/injury primarily in the chloroplasts due to the production of free radicals (Heber *et al.*, 1971; Santarius, 1987). Exposure to low temperatures has also been reported to inhibit the energy-consuming Calvin-Benson cycle enzymes more than the energy-transducing light reactions, thus promoting leakage of energy to oxygen (Wise, 1995). This in turn, presents the possibility of H_2O_2 production via oxidative reactions (Wise, 1995) (1.9.2) (Chapter 8). In both *V. sessilis* and *E. gracilis* inhibition of photosynthetic capacity was observed on chilling, cryoprotectant exposure and freezing, with lethal injury in *V. sessilis* induced by freezing, promoting lipid peroxidation. Exposure to high subzero temperatures, -60°C and LN also induced significant accumulation of lipids within the chloroplasts of *V. sessilis*, this may be due to stresses induced by exposure to low temperatures (Chapter 4). It was anticipated that lipid peroxidation would be detected in *E. gracilis*, however, the TBARS assay proved an inappropriate technique for this alga as interference from other cell components occurred (Chapter 8).

Chapters 3 and 5 highlighted the different responses of *H. pluvialis* and *E. gracilis* to chilling and freezing. Photosynthetic capacity in *H. pluvialis* [which was successfully cryopreserved at the CCAP (Day *pers. comm.*)] was not demonstrated to be inhibited following exposure to subzero temperatures. However, *E. gracilis* was observed to experience considerable inhibition of photosynthetic oxygen evolving capacity after exposure to subzero temperatures. The inhibition of photosynthesis, may, as detailed above, predispose *E. gracilis* to oxidative stress. It could promote the production and accumulation of excessive levels of H_2O_2, which may in turn initiate damage in *E. gracilis* by toxic hydroxyl radicals produced via Haber-Weiss/Fenton chemistry from H_2O_2.

The diverse morphology and physiology of algae clearly contribute to the difficulty in developing a general standardised cryopreservation protocol. This variation was also reflected in their antioxidant status/content, with some algae including *E. gracilis* apparently lacking catalase and relying on the rapid diffusion of H_2O_2 to the cytosol for its regulation, thus preventing toxic injury or its compliance in Haber-Weiss/Fenton chemistry (Chapter 8) (Brown *et al.*, 1975; Shigeoka *et al.*, 1980). This adaptation in *E. gracilis* may contribute to its comparative freeze recalcitrance, promoting the production of $^{\cdot}OH$ radicals during periods of high oxidative stress. In addition, the antioxidant compliment of the alga could contribute to injury, where imbalances in the complex interactions between antioxidants may permit the excessive accumulation of toxic metabolites, *e.g.*, H_2O_2 and GSSG (Chapter 8). In *E. gracilis*, chilling and freezing stresses induced responses in antioxidant status (Chapter 8). However, the greater increase in SOD activity in *E. gracilis* in response to oxidative stress, compared to *H. pluvialis*, may have resulted in an excessive accumulation of H_2O_2 (Chapter 8). *E. gracilis* was also found to contain elevated levels of other antioxidants, particularly those which serve to remove H_2O_2 (Chapter 8). However, these antioxidants may in themselves have increased oxidative injury in *E. gracilis* when poorly regulated (Chapter 8). Shigeoka *et al.* (1980) reported that *E. gracilis* lacked catalase and relied on the rapid diffusion of H_2O_2 into the cytosol, this mechanism may compound control of oxidative stresses (in excess of those encountered due to normal essential reactions) (Chapter 8). This may contribute to the difficulty in cryopreserving *E. gracilis*. The high

levels of ·OH radicals indirectly detected in *E. gracilis* cultures subjected to freeze-induced stress, using gas chromatography further supports the hypothesis. This implied that H_2O_2 production was not effectively regulated by those antioxidant enzymes which remove H_2O_2. There then exists potential for ·OH production via transition metal ion catalysed Haber-Weiss/Fenton chemistry in *E. gracilis* (Chapter 7, Chapter 1). Far from a lack of antioxidant response, it is possible that an additional mechanism contributing to cryoinjury in *E. gracilis* may be excessive antioxidant fluctuations which promoted imbalances in the cells antioxidant responses (Chapter 8) (Figs. 8.1-8.7). The differences between the algae which influence their susceptibility to injury also extends to their basic chemical composition including levels of protein SH groups, these protect the cell by serving as a preferential substrate for S-H oxidation (Chapter 8).

9.2.3 The biochemical approach to elucidating and preventing cryoinjury

Oxidative stress was clearly identified in *E. gracilis* (Chapter 7) by the indirect assay of the production of highly toxic hydroxyl radicals (·OH) using a novel non-destructive gas chromatgraphic technique. Cells were demonstrated to experience both lethal and non-lethal stresses. However, the alga was also demonstrated to be able to enhance its antioxidant status in response to cryoinjury (Chapter 8).

Developing the approach of Benson *et al.* (1995), investigations were performed into the use of the exogenous powerful iron chelator desferrioxamine to reduce oxidative stress/injury resulting from the production of hydroxyl radicals via Haber-Weiss/Fenton chemistry. Studies indicated that the addition of an exogenous iron chelating compound (10 $mg.l^{-1}$ desferrioxamine) could significantly reduce ·OH production in cultures of *E. gracilis* and thus enhance survival, post-thaw following exposure to LN ($F_{2,6} = 5.26$, $P < 0.05$). Further natural iron chelators have been applied to reduce oxidative injury in other systems (Kono *et al.*, 1998). The dietary antioxidant chlorogenic acid has been shown to be effective in inhibiting the iron-induced lipid peroxidation of bovine liver microsomes (Kono *et al.*, 1998). In addition, the accumulation of products of lipid peroxidation (malondialdehyde, conjugated dienes, lipid peroxides, and Schiff bases) in low temperature preserved kidney tissue, may be substantially reduced both during hypothermic storage and during the subsequent incubation period by addition of Trolox

(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; a water soluable analogue of α-tocopherol) (1 mM) or desferrioxamine (1 mM) as supplemental antioxidants (McAnulty & Haung, 1997). However, ascorbate (1 mM) was found to have prooxidant effect as a sole additive, or an extremely prooxidant effect when combined with either desferrioxamine or Trolox (McAnulty & Haung, 1997). Use of supplemental exogenous antioxidants may therefore be effectively employed in reducing oxidative stress in cryopreserved material, however, they must be selected carefully, and the complex interactions of antioxidants must be taken into account.

In addition to these specific exogenous antioxidants, novel encapsulation techniques may also provide cryoprotection by forming complexes with transition metal ions, possibly preventing their subsequent involvement in Haber-Weiss/Fenton chemistry (Chapter 6). Furthermore, many of the cryoprotectants employed have free radical scavenging capabilities (Benson & Withers, 1987) (Chapter 1).

9.3.1 Conclusions

The original data reported in this thesis demonstrate the feasibility, and value of, investigating mechanisms of cell damage induced during cryopreservation and employing the results to assist the development of improved cryopreservation protocols. The results testify the benefit of using investigative rather than speculative approaches to develop cryopreservation protocols. Many of the novel non-invasive techniques employed in this study may be further adapted for the evaluation of stress within other types of culture collections. The new data accrued has provided a valuable insight into cryoinjury in microalgae and as a result a new hypotheses of freeze-recalcitrance has been developed.

The biochemical basis of cryoinjury in algae appears to be due primarily to damage/disruption of the chloroplasts causing subsequent imbalances in antioxidant levels which in turn result in stress induced free radical damage. In *E. gracilis* cryoinjury may involve increased superoxide dismutase activity, which increases levels of H_2O_2, which then contribute to the generation of $^{\cdot}OH$ and the initiation of subsequent lipid peroxidation (Burdon *et al.*, 1994). Elevating levels of other antioxidants, when

poorly regulated, may also increase oxidative injury in *E. gracilis* (Chapter 8). More efficient and regulated antioxidant responses in *H. pluvialis*, combined with the high carotenoid composition of this alga may contribute to its greater cryo-tolerance.

During this research the application of a non-invasive technique for the investigation of free radical levels and the successful use of a range of antioxidant assays (following the development of a robust extraction procedure for cellular enzymes), has permitted improved understanding of the mechanisms of cell damage and recovery in the algae to be attained. The use of exogenous antioxidants and novel techniques (*e.g.*, alginate encapsulation) have also been demonstrated to have considerable potential permitting the successful cryopreservation of a wide range of protists.

9.3.2 Further work

It would be both interesting and useful to further develop the antioxidant studies performed during the course of this research to include a greater range of organisms. This would hopefully permit a more detailed understanding of fluctuations in antioxidant status in readily cryopreservable and freeze recalcitrant organisms. Further antioxidant studies, measuring fluctuations in ascorbate and glutathione peroxidases together with investigations into SOD activity on a Cu/ZnSOD, MnSOD and FeSOD unit basis could also assist in the development of more effective protocols. The use of the gas chromatography to investigate markers of free radical activity in additional algae, particularly *Vaucheria sessilis* and *Haematococcus pluvialis* and in cells preserved using encapsulation would be of interest. Furthermore, investigation of the, as yet, unidentified volatiles observed during the course of this study would be worthy of further attention.

The author also believes that the development of microscopy techniques to visualise events occurring during cryopreservation would provide further valuable insights into the mechanisms of cryoinjury and recovery. In particular; linking of cryomicroscopic techniques to emerging microscopical techniques (*e.g.,* confocal microscopy). Further ultrastructural studies and the development of techniques for the investigation of biochemical events within the cell (*e.g.*, immunogold-labelling) would also be useful.

A further area which merits work, is the application of exogenous chemicals to limit free radical injury/damage. During this research a protective effect was detected when an exogenous iron chelating compound (10 $mg.l^{-1}$ desferrioxamine) was introduced to reduce $^{\cdot}OH$ production (Chapter 7). However, further chelating compounds are available, as are exogenous sources of antioxidants (Kono *et al.*, 1998; McAnulty & Haung, 1997; Laughton *et al.*, 1991; Evans *et al.*, 1997). It would, therefore, be interesting to evaluate the use of these compound in protistan cryopreservation.

9.4 The future of protistan cryopreservation

As reported in this thesis, cryopreservation has already been successfully employed in a number of biological systems, permitting the long-term storage of viable material. The World Federation of Culture Collections (WFCC), the United Kingdom Federation of Culture Collections (UKFCC) and the European Culture Collection Organisation (ECCO) have all recognised the importance of cryopreservation in their diverse range of culture collections (Kirsop & Snell, 1984; Kirsop & Doyle, 1991). In addition, several of the largest protistan collections have begun to develop methods suitable for the long-term preservation of their holdings (1.12).

In Chapters 3 and 6 extended, studies confirmed that the use of cryopreservation was appropriate for the long-term conservation of protistan strains. These data were consistent with long-term viability studies reported by Day *et al.* (1997). Throughout this study, the morphological and physiological diversity of the algae was found to influence their responses to stresses and injury induced during cryopreservation protocols. Improved understanding of mechanisms of cryoinjury/damage and recovery in cryopreserved algae gained during this research presents the possibility of "designing out" injurious factors within the cryopreservation protocol itself and developing recovery procedures which assist post-thaw recovery (*e.g.*, through the use of exogenous antioxidants) by adopting a considered strategic approach to method development. The development of cryopreservation protocols where cultures are assessed for antioxidant response and markers of free radical attack/stress are likely to lead to improved methodology and subsequent increases viability levels and permit the cryopreservation

of presently recalcitrant protists. This will, hopefully, permit the routine application of low temperature storage in protistan collections.

Finally, there is an increasing requirement for cryopreservation of protists. Forces driving the necessity to develop routine cryopreservation protocols include:

- The need to conserve biodiversity (see Chapter 1).
- The requirements of the Convention on Biodiversity (see Chapter 1) and the guidelines for the deposit of microorganisms detailed under the Budapest Treaty (Anonymous, 1995).
- Continued and increasing biotechnological exploitation of cultured material (see Chapter 1).
- Increasing costs associated with the maintenance of large service collection by more conventional techniques (Day *et al.*, 1997) (see Chapter 1).
- The technical advances which now permit the development of routine cryopreservation procedures for a wide range of organisms, including those detailed in this thesis.

Appendices.

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Appendix 1. WDCM (1994) Registered Algal Culture Collections of the World

Country	No.[a]	Collection	Acronym
Argentina	2	Collection Catedra Microbiologia Agricola	CCMA
Australia	157	Murdoch Uni. Algal Culture Collection	MUR
	16	Australian Collection of Micro-organisms, U. of Queensland	ACM
	2	Microbial Teaching Collection, Roseworthy Agricultural Col.	RAC
	300	CSIRO Culture Collection of Microalgae	CSIRO
	4	The Australian Collection of Antarctic Micro-organisms	ACAM
	5	Culture Collection, U. New south Wales	UNSW
	5	Australian National Reference Library	AMMRL
		Australian Water Quality Centre	ACWQ
		Melbourne University Culture Collection	MUCC
		The Australian Collection of Marine Micro-organisms	ACCM
Austria	1600	Algensammlung am Institut für Botanik, Uni. Innsbruck	ASIB
Brazil		Culture Collection of Freshwater Microscopic Algae at the Federal U. of Sao-Carlos	
Bulgaria		Plovdiv Algal Culture Collection	PACC
Canada	1	National Research Council of Canada	NRC
	133	Culture Collection, Dept. Plant Science, U. of Western Ontario	UWO
	180	U. of Toronto Culture Collection	UTCC
	2	U. Alberia Microfungus Collection and Herbarium	UAMH
	340	North East Pacific Culture Collection, U. British Columbia	NEPCC
China	2	China Centre for Type Culture Collection, Wuhan Uni.	CCTCC
		Collection of Asian Phytoplankton	CAP
		Institute of Hydrobiology	JINAN
Czech.	140	Culture Collection of Algae	CAUP
	8	Czechoslovak Collection of Yeasts	CCY
	522	Culture Collection of Autotrophic Organisms	CCALA

Denmark		Scandinavian Culture Centre for Algae and Protoza	SCCAP
Egypt	20	Cairo Microbiological Centre	CAIRO
France	200	Pasteur Culture Collection of Cyanobacterial Strains in Axenic Culture	CAEN
		Centre d'Oceanologie de Marseille	COM
		Centre de Nants	CN
Germany	1400	Sammlung von Algenkulturen	SAG
	400	Sammlung von Conjugaten-Kulturen	SVCK
Greece		National Centre for Scientific Research	NCSR
		University of the Aegean Culture Collection	UACC
India	12	National Collection of Industrial Micro-organisms	NCIM
	3	Biological Nitrogen Fixation Project	MPKV
Indonesia	10	Indonesian Sugar Research Institute	ISRI
	25	Institute of Technology Bandung	ITBCC
	7	Biotechnology Culture Collection	BTCC
Israel		Israel Oceanographic and Limnological Research LTD	IOLR
Italy	58	Centro di Studio dei Micro-organismi	CSMA
Japan	500	Microbial Culture Collection NIES	NIES
	500	Institute of Applied Microbiology	IAM
		Akashiwo Research Institute of Kagawa Prefacture	KAGAWA
		Faculty of Agriculture U. of Tokyo	FAUT
		Kyoto University Culture Collection	KUCC
		Marine Biotechnology Institute, Kamaishi Laboratories	MBI
		Department of Marine Sciences	MSTU
Malaysia	300	Dept. of Biochemistry, University of Malaya	DBUM; IPT
Mexico	1	Pathogen Fungi and Actinomycetes Collection	INDRE
	15	Centro de Investigation y de Estudios	CDBB
	5	Collection de Cpas Microbianas	ITD
	94	Industrial Culture Collection, Inst. de Invesigaciones Biomedicas	IIBM-UNAM
		La Coleccion de Microalgas del CICESE	CICESE

New Zealand		New Zealand Oceanographic Institute Phytoplankton Culture Collection	NZOI
		The Cawthron Microalgae Culture Collection	CAWT
NIS	700	Peterhof Genetic Collection of Microalgae	PGC
(former USSR)	15	The Collection of Algae, Inst. for Biology of Inland Waters Academy	BOROK
	20	Russian Collection of Industrial Micro-organisms	VKPM
	600	Collection of Algal Cultures, Leningrad Uni.	
	600	Collection of Microalgae of the Institute of Plant Physiology	IPPAS
		Collection of Algal Strains, Division of Spore Plantae	IBASU-A
Norway	260	Culture Collection of Algae (NIVA)	NIVA
		University of Oslo, Dept. of Marine Biology	UIO
Pakistan	20	Pakistan Type Culture Collections	PTCC
Philippines	1	Industrial Technology Development Institute	ITDI
	20	Algal Culture Collection, Museum of Natural History	UPLB
	9	BIOTECH Microbial Culture Collection	BIOTECH
		Blue-Green Algal Collection	IRRI
		Marine Science Institute	MSIUP
Portugal		ACOI-The Culture Collection of Algae of the Department of Botany U. of Coimbra	
Saudi Arabia		King Fahd University of Petroleum & Minerals, Algal Collection	KFUPM
Senegal	10	Mircen Afrique Ouest, ISRA-CNRA	MAO
South Africa		Dept. Botany, U. of Witwatersrand Culture Collection	WITS
Spain		Instituto Espanol de Oceanografia	VIGO
Sri Lanka	5	Dept. Botany, Uni. of Jaffna	UJB
Taiwan		Tungkang Marine Laboratory, Taiwan Fisheries Institute	TML
Thailand	10	Dept. Biology, Khon Kaen Uni.	DBKKU2
	10	Bept. Biology, Chaing Mai Uni.	MSCMU
	15	Dept. Agriculture, Div. Plant Pathology	BSMB
	15	Ministry of Agriculture Co-operative	SMRG
	194	TISTR Culture Collection Bangkok, MIRCEN	TISTR
	2	Dept. App. Biology, King Mongkut's Inst. of Technology	ABKMI
	200	Inst. of Food Research and Product Development	IFRPD

	3	Div. Mycology, Mahidol Uni.	DMMU3
United Kingdom	1700	Culture Collection of Algae and Protozoa	CCAP
	150	Plymouth Culture Collection	PHBL
		Swansea Algal Research Unit, U. of Wales	SARU
		U. of Westminster Algal Collection	UW
USA	10	Agriculture Research Service Culture Collection	NRRL
	1000	Provasoli-Guillard National Centre for Culture of Marine Phytoplankton	CCMP
	120	American Type Culture Collection	ATCC
	165	Carolina Biological Supply Co.	LMS
	20	Algal Culture Facility, Dept. of Biology, Uni. S. Carolina	ACF
	2089	Culture Collection of Algae at the University of Texas at Austin	UTEX
	30	The Upjohn Culture Collection	UC (UPJOHN)
	8	Lilly Culture Collection, Eli Lilly and C.	LRL
		Algal Collection at Monterey Bay Aquarium Research Institute	MBARI
		Chlamydomonas Genetics Centre	CGC
		Marine Microalgae Research Culture Collection	FLORIDA
	225	Freshwater Diatom Culture Collection	FDCC
		Milford Laboratory Culture Collection	MIL
		University of California-Santa Barbara Culture Collection	UCSB
		University of Miami Algal Culture Colection	MIAMI
		University of Rhode Island	URI-1, 2
		Woods Hole Oceanographic Institution	WHOI-1,2
Vietnam		National Scientific Research Centre of Vietnam	

[a] Number of culture collections per country.

(Andersen, 1996; WDCM, 1994; Ma *et al.*, 1995; *pers. comm.* J. Day)

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